Detecting parameter regions for bistability in reaction networks

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Detecting parameter regions for bistability in reaction networks

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Abstract

Deciding whether and where a system of parametrized ordinary differential equations displays bistability, that is, has at least two asymptotically stable steady states for some choice of parameters, is a hard problem. For systems modeling biochemical reaction networks, we introduce a procedure to determine, exclusively via symbolic computations, the stability of the steady states for unspecified parameter values. In particular, our approach fully determines the stability type of all steady states of a broad class of networks. To this end, we combine the Hurwitz criterion, reduction of the steady state equations to one univariate equation, and structural reductions of the reaction network. Using our method, we prove that bistability occurs in open regions in parameter space for many relevant motifs in cell signaling.

1 Introduction

Bistability, that is, the existence of at least two stable steady states in a dynamical system, has been linked to switch-like behavior in biological networks and cellular decision making and it has been observed experimentally in a variety of systems \([25, 32, 33]\). However, proving the existence of bistability in a parameter-dependent mathematical model is in general hard.

We focus on (bio)chemical reaction networks with associated kinetics, giving rise to systems of Ordinary Differential Equations (ODEs) that model the change in the concentration of the species of the network over time. These systems come equipped with unknown parameters and, ideally, one wishes to determine properties of the family of ODEs for varying parameter values. Here we are concerned with stability of the steady states, and focus on the following three questions: (1) if the network admits only one steady state for all parameter choices, is it asymptotically stable? (2) can parameter values be chosen such that the system is bistable? (3) does it hold that for any choice of parameters yielding at least three steady states, two of them are asymptotically stable?

As the parameters are regarded unknown, explicit expressions for the steady states are rarely available. Problem (1) has been shown to be tractable for certain classes of networks. For example, the only steady state of complex balanced networks admits a Lyapunov function making it asymptotically stable \([18, 27]\). The use of Lyapunov functions and the theory of monotone systems has been employed more broadly to other classes of networks \([15, 1, 3]\). Finally, algebraic criteria as the Hurwitz criterion or the study of P-matrices also provide asymptotic stability of steady states, often in combination with algebraic parametrizations of the steady state variety. In \([9]\) the Hurwitz criterion is analyzed using graphical methods.

Problem (2) is much harder and typically tackled by first deciding whether the network admits multiple steady states using one of the many available methods \([30]\), and then numerically computing the steady states and their stability for a suitable choice of parameter values. Rigorous proofs of bistability require advanced analytical arguments such as bifurcation theory and geometric singular perturbation theory, as employed in \([26, 20]\) for futile cycles. The use of the Hurwitz criterion to prove bistability is anecdotal, as rarely explicit descriptions of the steady states can be found. Problem (3) has been addressed for small systems using case-by-case approaches, but no systematic strategy has been proposed.

We devise a flow chart to solve the problems (1)-(3) using computer-based proofs relying only on symbolic operations. This is achieved by combining three key ingredients. First, we apply the Hurwitz
modeled by means of a system of autonomous ODEs, \( A \) with rows (resp. columns) with indices in \( d \) of \( v \) kinetics species. We consider reaction networks over a set of \( A \), \( V \) complement of a vector space \( = \text{dim}(T) \), \([37]\). Furthermore, any trajectory of Eq. (2) is confined to a so-called stoichiometric compatibility class (invariant by Eq. (2), \([37]\). Third, if Theorem 2 does not apply, we reduce the network to a smaller one for which Theorem 2 applies. Afterwards, we infer stability properties of the steady states of the original network in an open parameter region. To this end, many reduction techniques have been proposed \([11, 23]\), but often removal of reactions \([29]\) and of intermediates \([24]\) suffice for (bio)chemical networks.

Even though our approach demands heavy symbolic computations, we illustrate how problem (3) can successfully be tackled for small networks, and further, we prove the existence of bistability in open regions of the parameter space, with the exception of double phosphorylation cycle, whose bistability was proven in \([26]\).

2 Mathematical framework

We use the following notation: \( J_f \) is the Jacobian matrix of a function \( f \). We denote by \( V^\perp \) the orthogonal complement of a vector space \( V \). For \( A \in \mathbb{R}^{n \times n} \) and \( I, J \subseteq \{1, \ldots, n\} \), we let \( A_{I,J} \) be the submatrix of \( A \) with rows (resp. columns) with indices in \( I \) (resp. \( J \)). Finally, we denote by \( p_A(\lambda) \) the characteristic polynomial of \( A \).

2.1 Reaction networks

We consider reaction networks over a set of species \( S = \{X_1, \ldots, X_n\} \) given by a collection of reactions

\[
 r_j : \sum_{i=1}^n \alpha_{ij} X_i \longrightarrow \sum_{i=1}^n \beta_{ij} X_i \quad \text{for } j = 1, \ldots, m \tag{1}
\]

with \( \alpha_{ij} \neq \beta_{ij} \) for at least one index \( i \). Let \( x_i \) denote the concentration of \( X_i \). Given a differentiable kinetics \( v : \mathbb{R}_{\geq 0}^n \rightarrow \mathbb{R}_0^m \), the dynamics of the concentrations of the species in the network over time \( t \) are modeled by means of a system of autonomous ODEs,

\[
 \frac{dx}{dt} = Nv(x), \quad x = (x_1, \ldots, x_n) \in \mathbb{R}_{\geq 0}^n, \tag{2}
\]

where \( N \in \mathbb{R}^{n \times m} \) is the stoichiometric matrix with \( j \)th column \( (\beta_{1j} - \alpha_{1j}, \ldots, \beta_{nj} - \alpha_{nj}) \). We write \( f(x) := Nv(x) \). With mass-action kinetics, we have \( v_j(x) = \kappa_j x_1^{\alpha_{1j}} \cdots x_n^{\alpha_{nj}} \) where \( \kappa_j > 0 \) is a rate constant, shown often as a label of the reaction.

Under mild conditions, satisfied by common kinetics including mass-action, \( \mathbb{R}_{\geq 0}^n \) and \( \mathbb{R}_{\geq 0}^m \) are forward invariant by Eq. (2), \([27]\). Furthermore, any trajectory of Eq. (2) is confined to a so-called stoichiometric compatibility class \( (x_0 + S) \cap \mathbb{R}_{\geq 0}^n \) with \( x_0 \in \mathbb{R}_{\geq 0}^n \), where \( S \) is the column span of \( N \) and called the stoichiometric subspace \([19]\). The set \( (x_0 + S) \cap \mathbb{R}_{\geq 0}^n \) is the solution set of the equations \( Wx = T \) with \( W \in \mathbb{R}^{d \times n} \) any matrix whose rows form a basis of \( S^\perp \) and \( T = Wx_0 \in \mathbb{R}^d \). These equations are called conservation laws and the defined stoichiometric compatibility class is denoted by \( \mathcal{P}_T \).

2.2 Steady states

The steady states (or equilibria) of the network are the non-negative solutions to \( Nv(x) = 0 \). The positive steady states, that is, the solutions in \( \mathbb{R}_{\geq 0}^n \), define the positive steady state variety \( V^+ \). The existence of \( d \) linearly independent conservation laws implies that \( d \) steady state equations are redundant. We let \( s = \text{dim}(S) \), \( d = n - s \), and \( W \in \mathbb{R}^{d \times n} \) be row reduced with \( i_1, \ldots, i_d \) the indices of the first non-zero coordinate of each row. For \( T \in \mathbb{R}^d \), define

\[
 F_T(x)_i = \begin{cases} f_i(x) & i \notin \{i_1, \ldots, i_d\} \\ (Wx - T)_i & i \in \{i_1, \ldots, i_d\}, \end{cases} \tag{3}
\]
which arises after replacing redundant equations in $Nv(x) = 0$ with $Wx = 0$. Hence, the solutions to $F_T(x) = 0$ are the steady states in $P_T$\cite{10,38}. A steady state $x^*$ is non-degenerate if $\ker(J_f(x^*)) \cap S = \{0\}$, or equivalently, if $\det(J_{F_T}(x^*)) \neq 0$\cite{35}. Observe that $J_{F_T}(x^*)$ is independent of $T$.

As an example, consider the following reaction network

$$X_1 \xrightarrow{\kappa_1} X_2 \quad X_2 + X_3 \xrightarrow{\kappa_2} X_1 + X_4 \quad X_4 \xrightarrow{\kappa_3} X_3.$$ \hspace{1cm} (4)

This is a simplified model of a two-component system, consisting of a histidine kinase HK and a response regulator RR\cite{10}. Both occur unphosphorylated ($X_1, X_3$) and phosphorylated ($X_2, X_4$). With mass-action kinetics, the ODE system is

$$\frac{dx_1}{dt} = -\kappa_1 x_1 + \kappa_2 x_2 x_3 \quad \frac{dx_2}{dt} = \kappa_1 x_1 - \kappa_2 x_2 x_3 \quad \frac{dx_3}{dt} = -\kappa_2 x_2 x_3 + \kappa_3 x_4 \quad \frac{dx_4}{dt} = \kappa_2 x_2 x_3 - \kappa_3 x_4,$$

and we consider $P_T$ defined by $x_1 + x_2 = T_1$ and $x_3 + x_4 = T_2$. With this choice, $F_T(x)$ is

$$(x_1 + x_2 - T_1, \kappa_1 x_1 - \kappa_2 x_2 x_3, x_3 + x_4 - T_2, \kappa_2 x_2 x_3 - \kappa_3 x_4).$$

In this example, the positive steady states are the positive solutions to the equations $\frac{dx_1}{dt} = \frac{dx_2}{dt} = \frac{dx_3}{dt} = 0$, which when solved for $x_1$ and $x_3$ lead to the following parametrization of $V^+$:

$$\phi(x_2, x_4) = \left(\frac{\kappa_2 x_4}{\kappa_1}, x_2, \frac{\kappa_2 x_4}{\kappa_2 x_3}, x_4\right), \quad (x_2, x_4) \in \mathbb{R}_+^2.$$ \hspace{1cm} (5)

In general, we refer to a positive parametrization as any bijective map of the form

$$\phi: \mathbb{R}_{>0}^d \to V^+ \quad \text{with} \quad \xi \mapsto \phi(\xi).$$ \hspace{1cm} (6)

In practice, under mass-action kinetics, the entries of $\phi$ are rational functions in $\xi$. Strategies for finding positive parametrizations are reviewed in\cite{10}.

### 2.3 Multistationarity and bistability

A network is multistationary if it has at least two positive steady states in some $P_T$, that is, $F_T(x) = 0$ has at least two positive solutions for some $T \in \mathbb{R}_4$. A monostationary network has exactly one positive steady state in each $P_T$. Under some conditions, if the sign of $\det(J_{F_T}(x^*))$ is $(-1)^s$ for all positive steady states $x^*$, then the network is monostationary; if the sign is $(-1)^{s+1}$ for some $x^*$, then it is multistationary\cite{10}. Using a positive parametrization, this result yields inequalities in the rate constants that guarantee or preclude multistationarity\cite{11,10}.

Given $\frac{dx}{dt} = f(x)$, a steady state $x^*$ is stable if for each $\epsilon > 0$ small enough, there exists $\delta > 0$ such that solutions starting within distance $\delta$ of $x^*$, remain within distance $\epsilon$. If additionally $\delta$ can be chosen such that solutions tend to $x^*$ as time increases, $x^*$ is asymptotically stable. If $x^*$ is not stable, then it is unstable. If all eigenvalues of $J_f(x^*)$ have negative real part, then $x^*$ is exponentially stable. Exponential stability implies asymptotic stability (§2.7-2.8 in\cite{35}). If one eigenvalue has positive real part, then $x^*$ is unstable. For further discussions on stability we refer to\cite{35}.

The stability of a steady state is studied relatively to $P_T$. A network that admits two asymptotically stable positive steady states in some $P_T$ is called bistable. Detecting multistationarity and bistability is challenging already for medium sized networks. To overcome computational difficulties one may employ structural modifications of the network, such as removal of intermediates or reactions. Specifically, given two networks $G$ and $G'$, $G'$ is a subnetwork of $G$ if it arises after removing reactions of $G$\cite{20}. An intermediate $Y$ is a species that appears only in the form $Y$ at either side of a reaction, and is both a product and a reactant in the network. Removal of intermediates leads to a new network after collapsing into one reaction all paths of reactions from and to non-intermediates and through intermediates\cite{23}.

For example, the species $S_0E$ in $S_0 + E \xrightarrow{\ell_1} S_0E \xrightarrow{\ell_2} S_1 + E$ is an intermediate. Its removal yields the reaction $S_0 + E \rightarrow S_1 + E$.

Assume mass-action kinetics and that $G'$ either is a subnetwork of $G$ such that both stoichiometric subspaces agree, or arises from $G$ by removal of intermediates and satisfies a technical condition on the rate constants\cite{23,39}. If $G'$ has $\ell_1$ exponentially stable and $\ell_2$ non-degenerate unstable steady states in.
some $P_T$ for some rate constants $\kappa$, then $G$ has at least $\ell_1$ exponentially stable and $\ell_2$ non-degenerate unstable steady states in some $P_T$ for some rate constants $\tilde{\kappa}$. If $G'$ is a subnetwork of $G$, then $\tilde{\kappa}$ agrees with $\kappa$ for the common reactions and is small enough for the reactions that only are in $G$. A more intricate but explicit description of the rate constants $\tilde{\kappa}$ exists also for the removal of intermediates [23].

2.4 The Jacobian matrix of reaction networks

In the context of reaction networks, we determine stability based on the eigenvalues of the Jacobian of the restriction of Eq. (2) to $P_T$. To this end, we consider the projection of $J_f(x)$ onto the stoichiometric subspace $S$ by writing the ODE system in local coordinates of $P_T$. Let $R_0 \in \mathbb{R}^{n \times n}$ be a matrix whose columns form a basis of $S$ and $L \in \mathbb{R}^{n \times m}$ such that $N = R_0L$. Then the projection of $J_f(x)$ onto $S$ is $LJ_v(x)R_0$.

**Proposition 1.** The characteristic polynomials $p_{J_f(x)}$ and $p_{LJ_v(x)R_0}$ satisfy

$$p_{J_f(x)}(\lambda) = \lambda^{n-s}p_{LJ_v(x)R_0}(\lambda).$$

Further, the independent term of $p_{LJ_v(x)R_0}(\lambda)$ is $(-1)^s \det(J_{F_\varphi}(x))$, with $F_T$ as in Eq. (3) for any choice of $W$.

The proof of Proposition 8 can be found in [A]. According to Proposition 8, the $s$ eigenvalues of $Q_x := LJ_v(x)R_0$ are the eigenvalues of $J_f(x)$ once zero counted with multiplicity $d$ is disregarded. In order to study the (sign of the real part of the) spectrum of the matrices $Q_x$ when $x$ is a positive steady state, we use a positive parametrization. We denote the characteristic polynomial $p_{Q_x}(\lambda)$ by $q_x(\lambda)$.

For example, for the network in Eq. (4), we consider the matrices $R_0, L, J_v(x)$ respectively to be

$$
\begin{bmatrix}
-1 & 0 & 1 & 0 \\
1 & 0 & 1 & 0 \\
0 & 1 & -1 & 0 \\
0 & 0 & 0 & 1
\end{bmatrix},
\begin{bmatrix}
1 & 0 & 0 & 0 \\
0 & -1 & 0 & 0 \\
0 & 0 & -1 & 0 \\
0 & 0 & 0 & 1
\end{bmatrix},
\begin{bmatrix}
\kappa_1 & 0 & 0 & 0 \\
0 & \kappa_2 & 0 & 0 \\
0 & 0 & \kappa_3 & 0 \\
0 & 0 & 0 & \kappa_4
\end{bmatrix},
$$

and we are interested in the eigenvalues of the matrix

$$Q_x = LJ_v(x)R_0 = \begin{bmatrix}
-\kappa_1 & \kappa_2 & 0 & 0 \\
\kappa_2 & -\kappa_3 & 0 & 0 \\
0 & 0 & -\kappa_2 & 0 \\
0 & 0 & 0 & -\kappa_3
\end{bmatrix}$$

(7)
evaluated at a steady state $x^* = \phi(x_2, x_4)$. Thus, by analyzing the eigenvalues of $Q_\varphi(x_2, x_4)$ for all values of $\kappa$ and $x_2, x_4 > 0$, we study the stability of all positive steady states.

We conclude this part with a key technical result (proven in [A]) on the determinant of $J_f(x^*)$ in the particular case where system $F_T(x)$ is reduced to one univariate equation.

**Proposition 2.** Fix $W \in \mathbb{R}^{m \times n}, T \in \mathbb{R}^d$ and $F_T$ as in Eq. (3). Assume there exist an open interval $E \subseteq \mathbb{R}$, a differentiable function $\varphi : E \rightarrow \mathbb{R}_{\geq 0},$ and indices $i, j$ such that $\varphi'(z) \neq 0$ and $F_{T,\ell}(\varphi(z)) = 0$ for all $\ell \neq j$. Then, for any solution $z^*$ to

$$F_{T,j}(\varphi(z)) = 0, \quad z \in E,$$

(8)
dej^+_{\varphi(z)} \det(J_{F_T}(x^*)^{-1}) = (F_{T,j} \circ \varphi)'(z^*) \det(J_{F_T}(x^*)_{J,I}),$$

where $I = \{1, \ldots, n\} \setminus \{i\}$ and $J = \{1, \ldots, n\} \setminus \{j\}$.

In practice, $\varphi(z) = z, \varphi \in \mathbb{R}_{\geq 0}$, and the solutions to $F_T(x) = 0$ are in one to one correspondence with the solutions to Eq. (4). In this case, given the positive solutions $z_1 < \cdots < z_{\ell}$ of Eq. (9), the sign of the derivative of $F_{T,j}(\varphi(z))$ evaluated at $z_1, \ldots, z_{\ell}$ alternates if all the steady states are non-degenerate. If additionally the sign of $\frac{1}{\varphi'(z)} \det(J_{F_T}(\varphi(z))_{J,I})$ is independent of the choice of $z$, then the sign of $\det(J_{F_T}(\varphi(z)))$ depends only on the sign of the derivative of $(F_{T,j} \circ \varphi)$ at $z_{\ell}$. We will exploit this fact below.
2.5 Algebraic criteria for stability

We present now the Hurwitz criterion [5, 2], which determines whether all the roots of a polynomial have negative real part.

**Criterion 1** (Hurwitz). Let \( p(x) = a_s x^s + \ldots + a_0 \) be a real polynomial with \( a_s > 0 \) and \( a_0 \neq 0 \). The Hurwitz matrix \( H = (h_{ij}) \) associated with \( p \) has entries \( h_{i,j} = a_{s-2i+j} \) for \( i, j = 1, \ldots, s \), by letting \( a_k = 0 \) if \( k \notin \{0, \ldots, s\} \):

\[
H = \begin{bmatrix}
a_{s-1} & a_s & 0 & 0 & \cdots & 0 \\
a_{s-3} & a_{s-2} & a_{s-1} & a_s & \cdots & 0 \\
\vdots & \vdots & \vdots & \vdots & \ddots & \vdots \\
0 & 0 & 0 & a_{s-s} & \cdots & a_2 \\
0 & 0 & 0 & 0 & \cdots & a_0
\end{bmatrix} \in \mathbb{R}^{s \times s}.
\]

The \( i \)th Hurwitz determinant is \( H_i = \det(H_{i,i}) \), \( I = \{1, \ldots, i\} \). Then, every root of \( p \) has negative real part if and only if \( H_i > 0 \) for all \( i = 1, \ldots, s \). If \( H_i < 0 \) for some \( i \), then a root of \( p \) has positive real part.

Note that \( H_s = a_0 H_{s-1} \). Pairs of imaginary roots (leading to Hopf bifurcations) arise when at least one of the \( H_i \) vanish [39] (see [12] in the context of reaction networks).

For the polynomial \( q_0(\lambda) \) or \( q_0(\xi)(\lambda) \), the Hurwitz determinants are typically rational functions in \( \lambda \) or \( \xi \). In Example in Eq. (4), the polynomial \( q_0(\lambda) \) for \( Q_s \) as in Eq. (7) is

\[
\lambda^2 + (\kappa_2 x_2 + \kappa_3 x_3 + \kappa_1 + \kappa_3) \lambda + \kappa_1 \kappa_2 x_2 + \kappa_2 \kappa_3 x_3 + \kappa_1 \kappa_3,
\]

and the Hurwitz determinants are \( H_1 = \kappa_2(x_2 + x_3) + \kappa_1 + \kappa_3 \) and \( H_2 = ((\kappa_1 x_2 + \kappa_3 x_3)\kappa_2 + \kappa_1 \kappa_3)H_1 \). Both determinants are polynomials in \( \kappa, x \) with positive coefficients and hence positive for all \( \kappa, x \in \mathbb{R}^+ \).

By the Hurwitz criterion any positive steady state is exponentially stable. This network has exactly one steady state in each \( \mathcal{P}_T \) [10], and we now additionally conclude that the only steady state is exponentially stable.

Often for small networks, all but the last Hurwitz determinants are positive. Then, the stability of a steady state \( x^* \) is fully determined by the sign of \( H_s \), which agrees with the sign of the independent term of \( q_s(\lambda) \), which in turn is \((-1)^s \det(J_{F_T}(x^*))\) by Proposition 3. Together with Proposition 4 we obtain the following theorem, proved in Appendix A.

**Theorem 1.** Let \( T, E, \varphi, i, j, I, J \) be as in Proposition 4. Assume that

- the sign of \( \frac{1}{\varphi'(z)} \det(J_{F_T}(\varphi(z)))_{I,I} \) is independent of \( z \in E \) and is nonzero, and

- the first \( s-1 \) Hurwitz determinants of \( q_{s-r}(\lambda) \) are positive for all positive steady states \( x^* \).

If \( z_1 < \cdots < z_\ell \) are the positive solutions to Eq. (9) and all are simple, then either \( \varphi(z_1), \varphi(z_2), \ldots \) are exponentially stable and \( \varphi(z_2), \varphi(z_4), \ldots \) are unstable, or the other way around. Specifically, \( \varphi(z_1) \) is exponentially stable if and only if

\[
\frac{(-1)^{s+r+j}}{\varphi'(z_1)} (F_{T,j}(\varphi(z_1))^1 \det(J_{F_T}(\varphi(z_1)))_{I,J}) > 0.
\]

In practice, \( F_{T,j}(\varphi(z)) = \frac{a(z)}{b(z)} \) is a rational function in \( z \) with \( b(z) > 0 \) in \( E \). Then the zeros of \( F_{T,j}(\varphi(z)) \) are the roots of \( a(z) \), and the signs of \( F_{T,j}(\varphi)'(z^*) \) and \( a'(z^*) \) agree for all \( z^* \in E \) such that \( a(z^*) = 0 \) (Lemma 1 in A).

We illustrate Theorem 3 with a hybrid histidine kinase network with mass-action kinetics [31], see Fig. 1. We rename the species as follows: \( X_1 = \text{HK}_00 \), \( X_2 = \text{HK}_p0 \), \( X_3 = \text{HK}_p0 \), \( X_4 = \text{HK}_p^p \), \( X_5 = \text{Htp} \) and \( X_6 = \text{Htp}_p \). The associated ODE system is

\[
\begin{align*}
\frac{dx_1}{dt} &= -\kappa_1 x_1 + \kappa_4 x_3 x_5 \\
\frac{dx_2}{dt} &= \kappa_1 x_1 - \kappa_2 x_2 + \kappa_5 x_4 x_5 \\
\frac{dx_3}{dt} &= -\kappa_3 x_3 + \kappa_2 x_2 - \kappa_4 x_3 x_5 \\
\frac{dx_4}{dt} &= -\kappa_3 x_3 - \kappa_2 x_2 + \kappa_4 x_3 x_5 \\
\frac{dx_5}{dt} &= \kappa_3 x_3 - \kappa_5 x_4 x_5 \\
\frac{dx_6}{dt} &= -\kappa_4 x_3 x_5 - \kappa_5 x_4 x_5 + \kappa_6 x_6.
\end{align*}
\]
The conservation laws of the system are $T_1 = x_1 + x_2 + x_3 + x_4$, $T_2 = x_5 + x_6$. Hence

$$F_T(x) = \begin{pmatrix} x_1 + x_2 + x_3 + x_4 - T_1 \\ \kappa_5 x_4 x_5 + \kappa_1 x_1 - \kappa_2 x_2 \\ -\kappa_4 x_3 x_5 + \kappa_2 x_2 - \kappa_3 x_3 \\ -\kappa_5 x_4 x_5 + \kappa_3 x_3 \\ x_5 + x_6 - T_2 \\ \kappa_4 x_3 x_5 + \kappa_5 x_4 x_5 - \kappa_6 x_6 \end{pmatrix}.$$

Here $s = 4$. The existence of three positive steady states for this network was established in [31]. We compute $q_s(\lambda)$ and the Hurwitz determinants in Maple 2019 and obtain that all but the last are positive. Since the denominator of \( \frac{\kappa_i}{\kappa_j} \) is a rational function with all coefficients positive, we are in the situation of Theorem 2. Since the independent term of the numerator of \( F_{T,5} \circ \varphi(z) \) is negative, its first root has positive derivative. Further, the sign of \( \frac{(-1)^{s+i+j}}{\varphi'(z_1)} \) is positive as well. We conclude, using Theorem 2, that whenever the network has three positive steady states coming from the roots $z_1 < z_2 < z_3$ of \( F_{T,5} \circ \varphi(z) \), then the steady states $\varphi(z_1)$ and $\varphi(z_3)$ are exponentially stable and the steady state $\varphi(z_2)$ is unstable. We have shown that this network displays bistability whenever there are three positive steady states.

### 3 Symbolic determination of stability

We now combine the ingredients introduced in the previous section into a strategy to determine the stability of positive steady states and, importantly, detect bistability, using (mainly) the Hurwitz criterion and Theorem 2. Given a reaction network with kinetics $v(x)$ the steps taken are depicted in Fig. 2. Specifically, we find $q_s(\lambda)$ and the Hurwitz determinants. If all determinants are positive, then the positive steady states are exponentially stable. If only the last Hurwitz determinant can be negative, then we attempt to apply Theorem 2. We only find a parametrization $\phi$ as in Eq. (6) when the sign of $H_i$ is not determined for arbitrary positive $x$. 

Figure 1: Three networks where stability of steady states is fully determined.
If some of the steps fail, then we consider reduced networks by removing either reactions that do not change the stoichiometric subspace, or intermediates. If stability is determined for a reduced network, then we conclude that the original network has at least the same number of positive steady states and stability properties as the reduced network in an open parameter region. In particular, if the reduced network has bistability, then so does the original network.

We now use this approach on the remaining networks in Fig. 4. We consider a two substrate enzyme catalysis mechanism, comprising an enzyme $E$ that binds two substrates, $S_1$ and $S_2$, and catalyzes the reversible conversion to $P$. Taken with mass-action kinetics this network has one positive steady state in each $P_T$ for any $\kappa > 0$. All but the last of the four Hurwitz determinants are positive for $x \in \mathbb{R}_{>0}$. We find a positive parametrization $\phi$ by solving the steady state equations in the concentrations of $ES_1$, $ES_2$, $ES_1S_2$ and $P$ using the procedure in [22], see Appendix C. After evaluating at $\phi$, $H_4$ becomes a rational function with only positive coefficients. Hence, all Hurwitz determinants are positive at a positive steady state, showing that the only positive steady state is exponentially stable.

Next, we consider the gene transcription network in Fig. 4 with mass-action kinetics. For any choice of rate constants there exist at least two positive steady states in some $P_T$ [10]. The computation of the Hurwitz determinants for arbitrary $x \in \mathbb{R}_{>0}$ gives that only $H_1, H_2$ are positive, but after evaluating at a positive parametrization, all but the last Hurwitz determinants are positive. We proceed to verify the assumptions of Theorem 2, see Appendix C. We obtain that the maximum number of positive steady states in any $P_T$ is 3, and that, whenever the network has one positive steady state, then it is exponentially stable, and if it has three positive steady states, then two of them are exponentially stable and one is unstable.

### 3.1 Bistability in cell signaling

After having illustrated our approach with selected examples, we now investigate relevant cell signaling motifs. All networks in Fig. 3 are known to be monostationary under mass-action [21]. All Hurwitz

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**Figure 3:** Monostationary networks. In all networks, the symbols $E, F, S, P$ refer to kinases, phosphatases, and substrates respectively. Taken with mass-action kinetics, all networks admit exactly one positive steady state in $P_T$, which further is exponentially stable. Networks (1)-(4) model the phosphorylation of one substrate via different mechanisms. Network (5) models a substrate with two phosphorylation sites, while network (6) models the phosphorylation of two different substrates.
determinants of $q_2(\lambda)$ are positive for positive $x$, without the need of a positive parametrization, see Appendix C. Hence for any rate constants, each network in Fig. 3 has exactly one positive steady state in each $P_T$, which further is exponentially stable.

In the examples so far, we have not employed network reduction techniques, because all steps of the method could be carried through and the stability of a steady state depended only on the sign of the determinant of the Jacobian. This scenario is quite restrictive, as it implies that instabilities arise from a unique eigenvalue with positive real part. The networks in Fig. 4 are all known to be multistationary with mass-action [21, 24]. Our method fails on the original networks: for network (c), the computation of the Hurwitz determinants was not possible in a regular PC due to lack of memory, and for the rest of the networks $H_i > 0$ for $i \neq s$ does not hold. However, Theorem 2 applies to the reduced networks in Fig. 4. In particular, all reduced networks in Fig. 4 display bistability whenever they have three positive steady states. Hence, after lifting stability to the original network, for all networks in Fig. 4 there is an open parameter region where the network has two exponentially stable positive steady states.

4 Computational challenges

In our context the Hurwitz determinants are symbolic and depend on $\kappa$ and $x$ or $\xi$. Their computation requires the storage of functions with many terms, which easily becomes infeasible in a regular PC. For example, for network (6) in Fig. 3 $H_4$ and $H_5$ are polynomials in $\kappa$ and $x$ with respectively 1,732,192 and 37,609,352 monomials, before the evaluation at a parametrization.

For medium sized networks some tricks can be applied under mass-action. A first strategy is to parametrize the positive steady state variety using convex parameters introduced by Clarke [9,17]. This conversion may reduce the number of parameters, mainly if the network has few reversible reactions.

The second strategy encodes a monomial $\eta x_1^{a_1} \cdots x_n^{a_n}$ as an $(n+1)$-tuple $(\eta, a_1, \ldots, a_n)$, and exploits relations among the $H_i$ obtained by expanding recursively along the last column, e.g. $H_3 = a_{s-3}H_2 - a_{s-1}(a_{s-1}H_1 + a_s a_{s-5})$ for a generic polynomial. Assume $H_1$ is written as a sum of terms that can be computed. We gather the $(n+1)$-tuples of each term into a list $L_1$ and create a list $L_2$ of the exponents.

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Figure 4: Multistationary networks and reductions to assert bistability. (a) $E_1$ and $E_2$ are two conformations of a kinase that catalyzes the phosphorylation of $S_0$ [22]. The reduced network is obtained by removing the intermediate $E_2S_0$ and all reverse reactions. (b) A cascade of two one-site modification cycles with the same phosphatase $F$. The reduced network is obtained by removing the intermediates $S_0E$ and $S_1F$ and all reverse reactions. (c) A cascade of one-site modification cycles with the same kinase $E$ and phosphatase $F$. The reduced network is obtained by removing the reverse reactions and intermediates $S_0E$, $S_1F$, $P_0S_1$, and $P_1F_2$. (d) Distribute and sequential phosphorylation of a substrate. The reduced network is obtained by removing the intermediates $S_1E$, $S_2F$ and $S_1F$ and all reverse reactions. (e) Phosphorylation of two substrates by the same kinase and phosphatase. The reduced network is obtained by removing the intermediates $S_0E$ and $P_1F$ and all reverse reactions. (f) Phosphorylation of a substrate with two sites catalyzed by the same kinase and two different phosphatases. The reduced network is obtained by removing all reverse reactions and the intermediates $S_1F_1$ and $S_2F_2$. 

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for which η is not a positive function. For each element in \( L_2 \), we consider the tuples in \( L_1 \) with that exponent, sum the coefficients and inspect the sign. If all coefficients are nonnegative, then so is \( H_i \). This procedure requires substantially less memory, but it might take time as lists are long. With this strategy we have determined the sign of \( H_3 \) in networks (5) and (6) in Fig. 4.

In special situations, \( H_i \) can be computed for \( x \), but evaluating at a parametrization and expanding the resulting polynomial to inspect its sign encounters memory issues. This problem can be addressed if there exists a monomial positive parametrization of the form \( \phi(\xi) = \alpha \circ \xi^B \) with \( B \in \mathbb{Z}^{d \times n} \) such that \( \phi(\xi)_i = \alpha_i(\xi^B)_i = \alpha_i \xi_1^{b_{i1}} \cdots \xi_n^{b_{in}} \), with \( \alpha \) depending on the rate constants [34]. In this case, a monomial \( \eta x_1^{a_1} \cdots x_n^{a_n} \) of \( H_i \) becomes \( \eta \alpha_1^{a_1} \cdots \alpha_n^{a_n} \xi^B_\alpha \). Hence, we first write all monomials as an \((n + d + 1)\)-tuple and record the evaluation with a new \((n + d + 1)\)-tuple \((\eta, a_1, \ldots, a_n, (Ba)_1, \ldots, (Ba)_d)\). We proceed as before to group the tuples and study the sign of the coefficients. With this approach, we computed the Hurwitz determinants of networks (a), (b), (d) and (e) in Fig. 4. However, \( H_{s-1} \) is not positive and Theorem 2 does not apply.

To verify that a polynomial with both positive and negative coefficients attains both signs, one can study the associated Newton polytope, as employed in the context of reaction networks in [10, 12] to cite a few. To assert that a polynomial only attains positive values despite having negative coefficients, one can employ techniques from sum-of-squares [7] and polynomial optimization via sums of nonnegative circuit polynomials [10, 28]. However, the size of the polynomials we encounter make these approaches challenging.

5 Discussion

All the steps of our procedure to determine the stability are symbolic and therefore provide computer-assisted proofs for bistability. In the most favorable scenario where Theorem 3 applies, the number of unstable and exponentially stable steady states is completely determined, and question (3) in the Introduction is answered. In particular, if the reduced univariate equation has at least three solutions and the first steady state is exponentially stable, the parameter region of bistability agrees with the parameter region giving three positive steady states. Finding the latter poses a simpler (though still hard) challenge, which can be addressed using recent methods [10, 6].

Under mass-action kinetics, reduction of the steady state equations to one polynomial can in principle be achieved using Groebner bases and invoking the Shape Lemma [13]. However, positivity is not addressed and the interval \( \varepsilon \) in Proposition 4 is rarely explicit. Reduction to one polynomial arises often after exploiting the inherent linearity the equations have [22].

In our procedure, the Hurwitz criterion can be replaced by other criteria of algebraic nature, namely the Liénard-Chipart criterion in [14] or checking whether the matrix \( Q_x \) is both a P-matrix and sign-symmetric. However, these criteria can only be used to assert exponential stability (see Appendix B where these criteria are applied to the network in Eq. (1)).

We have illustrated with numerous realistic examples that our approach determines bistability after performing network reduction. To our knowledge, this is a new result for all networks in Fig. 4 but network (d). For this one, bistability was formally proven in [26] using methods from geometric singular perturbation theory and the accurate study of a reduced network. We see our approach as a big step towards the automatic detection of bistability in open parameter regions, which relies on purely algebraic manipulations instead of advanced analytic arguments. Although the approach is applicable to arbitrary ODE systems, the special structure of the systems arising from reaction networks, specifically linearity, the existence of conservation laws and reduction techniques, make the approach particularly suited for this scenario.

Methods. All computations were made in Maple 2019. We first compute the Hurwitz determinants of a generic degree \( n \) polynomial and then evaluate at the coefficients of \( q_\alpha(\lambda) \). We disregarded the Routh table from the package DynamicSystems for using more memory.

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References


Appendices

In the Appendix A we provide the proofs of the results in the main text, in Appendix B expand on the stability criteria, and finally in Appendix C provide the details of the examples.

A Proofs of the results in the main text

We prove here the results in the main text. For completeness, we include the notation and the statements here, sometimes in an expanded form.

A.1 Proof of Proposition 3

Consider a matrix $R_0 \in \mathbb{R}^{n \times s}$ whose columns form a basis of $S$. This basis gives the system of coordinates in $S$. Therefore, given coordinates $z = (z_1, \ldots, z_s)$ in $S$, the vector $R_0 z$ is the vector of coordinates in the canonical basis of $\mathbb{R}^n$. Conversely, selecting a matrix $R_1 \in \mathbb{R}^{s \times s}$ such that $R_1 R_0 = I_{s \times s}$, we can write a vector $x \in S$ given in the canonical basis of $\mathbb{R}^n$, as a vector in local coordinates, by doing the product $R_1 x$.

Using these matrices, the ODE system restricted to $(x^* + S) \cap \mathbb{R}^n_{>0}$ in local $S$ coordinates is

$$\dot{z} = R_1 f(R_0 z + x^*)$$

after translating the steady state $x^*$ to the origin. The Jacobian matrix associated with this system at 0 is $R_1 J_f(x^*) R_0$. The following proposition shows some basic properties of $R_1 J_f(x^*) R_0$.

**Proposition 3.** Consider a reaction network with rate function $f(x) = N v(x)$. Let $R_0 \in \mathbb{R}^{n \times s}$ and $R_1 \in \mathbb{R}^{s \times n}$ be matrices such that the columns of $R_0$ form a basis of the stoichiometric subspace $S$, and $R_1 R_0 = I_{s \times s}$. The following statements hold:

- $R_1 J_f(x^*) R_0 = L J_0(x^*) R_0$, where $L \in \mathbb{R}^{s \times m}$ is the matrix such that $N = R_0 L$. In particular, $R_1 J_f(x^*) R_0$ does not depend on the choice of $R_1$.
- If $R_0, R'_0 \in \mathbb{R}^{n \times s}$ are two matrices with column span $S$, and $L, L'$ are as in (i) for $R_0, R'_0$, respectively, then the matrices $L' J_0(x^*) R'_0$ and $L J_0(x^*) R_0$ are similar.
- For a positive steady state $x^*$, the characteristic polynomials $p_{J_f} (\lambda)$ and $p_{L J_0(x^*) R_0} (\lambda)$ satisfy $p_{J_f} (\lambda) = \lambda^{n-s} p_{L J_0(x^*) R_0} (\lambda)$ for any choice of $R_0$.
- The independent term of $p_{L J_0(x^*) R_0} (\lambda)$ (or the coefficient of degree $n-s$ of $p_{J_f} (\lambda)$) equals $(-1)^s \det(J_{F_f}(x^*))$, with $F_f$ given in the main text, for any choice of row-reduced matrix of conservation laws $W$.

**Proof.** (i) Since the columns of $N$ belong to $S$, we can uniquely write $N = R_0 L$ with $L \in \mathbb{R}^{s \times m}$. Given that $J_f(x) = N J_v(x)$, we have

$$R_1 J_f(x^*) R_0 = R_1 N J_v(x^*) R_0 = R_1 R_0 L J_0(x^*) R_0 = L J_0(x^*) R_0.$$ 

(ii) Let $M \in \mathbb{R}^{s \times s}$ be the matrix of change of basis from $R_0$ to $R'_0$ such that $R_0 M = R'_0$. From $N = R'_0 L' = R_0 L$, it follows that $R_0 M L' = R'_0 L$ and thus $L' = M^{-1} L$. This gives

$$L' J_0(x^*) R'_0 = M^{-1} L J_0(x^*) R_0 M,$$

which implies that $L' J_0(x^*) R'_0$ and $L J_0(x^*) R_0$ are similar.

(iii) Extend the matrix $R_0$ to a square matrix $R \in \mathbb{R}^{n \times n}$ by adding columns such that $R$ has full rank $n$. Then the eigenvalues of the matrices $Q = R^{-1} J_f(x^*) R$ and $J_f(x^*)$ coincide. We choose $R_1$ as the first $s$ rows of $R^{-1} = \begin{bmatrix} R_1' \ R_1'' \end{bmatrix}$. Then $R_1 R_0 = I_{s \times s}$ and $R_1' R_0 = 0$. Since $\text{Im}(J_f(x^*)) \subseteq S$, the column span of $J_f(x^*)$ and $R_0$ agree, and hence $R_1' J_f(x^*) = 0$. Then, the matrix $Q$ is for the form

$$Q = \begin{pmatrix} R_1' & R_1'' \end{pmatrix} J_f(x^*) (R_0 \ R'_0) = \begin{pmatrix} R_1 J_f(x^*) \ 0 \end{pmatrix} (R_0 \ R'_0) = \begin{pmatrix} R_1 J_f(x^*) R_0 & R_1 J_f(x^*) R'_0 \ 0 & 0 \end{pmatrix}.$$
Clearly, the characteristic polynomial $p_Q(\lambda)$ is equal to $\lambda^{n-s}p_{R_1J_f(x^*)R_0}(\lambda)$. Using $R_1J_f(x^*)R_0 = LJ_{\phi}(x^*)R_0$, this concludes the proof of (iii), (iv) was proven in [35], Proposition 5.3.

\[\square\]

### A.2 Proof of Proposition 4

We now turn into the proof of Proposition 4, whose statement we recall here.

**Proposition 4.** Let $W \in \mathbb{R}^{d \times n}, T \in \mathbb{R}^d$ be fixed and consider $F_T$ as in the main text. Assume that there exist an open interval $\mathcal{E} \subseteq \mathbb{R}$, a differentiable function $\phi: \mathcal{E} \to \mathbb{R}^n_0$, and indices $i, j$ such that $F_{T,\ell}(\phi(z)) = 0$ for all $\ell \neq j$ and $\phi_i'(z) \neq 0$. Then, the set of positive solutions of the system $F_T(x) = 0$ contains the solutions to

$$F_{T,j}(\phi(z)) = 0, \quad x_\ell = \phi_i(z), \quad \ell = 1, \ldots, n \text{ and } z \in E,$$

and for a positive steady state $x^* = \phi(z)$ it holds

$$\det(J_{F_T}(x^*)) = \frac{(-1)^{i+j}}{\phi_i'(z)}(F_{T,j} \circ \phi)'(z) \det(J_{F_T}(x^*), \{j\}),$$

where $I = \{1, \ldots, n\} \setminus \{i\}$ and $J = \{1, \ldots, n\} \setminus \{j\}$.

**Proof.** In order to prove this identity, we make first three observations that rely on the definition of $\phi$ and on the chain rule for multivariate functions:

1. By hypothesis, $\phi(z) = (\phi_1(z), \ldots, \phi_n(z))$. Therefore, $\phi'(z) = (\phi_1'(z), \ldots, \phi_n'(z))$.
2. Since $F_{T,\ell}(\phi(z)) = 0$ for all $\ell \neq j$, we have that $(F_T \circ \phi)(z)$ is a vector with zeros in every entry except for the $j$-th entry, which is equal to $(F_{T,j} \circ \phi)(z)$. This implies that $(F_T \circ \phi)'(z)$ is also a vector with zero in every entry except in the $j$-th, that is equal to $(F_{T,j} \circ \phi)'(z)$.
3. By the chain rule $(F_T \circ \phi)'(z) = J_{F_T}(\phi(z))\phi'(z)$.

From observations (2) and (3) we have that

$$J_{F_T}(\phi(z))\phi'(z) = (0, \ldots, 0, (F_{T,j} \circ \phi)'(z), 0, \ldots, 0)^{tr},$$

which means that the linear combination of the columns of $J_{F_T}(\phi(z))$ given by the entries of $\phi'(z)$ is equal to the vector on the right side of the equation. Now, using observation (1), we compute $\det(J_{F_T}(x^*))$.

Indeed, denoting by $J_{F_T}^i$ the $i$-th column of $J_{F_T}$, [10] gives

$$\phi_i'(z)J_{F_T}^i(\phi(z)) = (0, \ldots, 0, (F_{T,j} \circ \phi)'(z), 0, \ldots, 0)^{tr} - \sum_{k=1, k \neq i}^n \phi_k'(z)J_{F_T}^k(\phi(z)).$$

Let $\tilde{J}_{F_T}(x^*)$ be the matrix obtained by replacing the $i$-th column of $J_{F_T}(\phi(z))$ by the vector

$$\left(0, \ldots, 0, \frac{(F_{T,j} \circ \phi)'(z)}{\phi_i'(z)}, 0, \ldots, 0\right)^{tr}.$$

Then, $\det(J_{F_T}(\phi(z)))$ and $\det(\tilde{J}_{F_T}(\phi(z)))$ agree. Now, expanding the determinant of $\tilde{J}_{F_T}(\phi(z))$ along the $i$-th column gives

$$\det(J_{F_T}(x^*)) = \frac{(-1)^{i+j}}{\phi_i'(z)}(F_{T,j} \circ \phi)'(z) \det(J_{F_T}(x^*), \{j\}).$$

This concludes the proof. \[\square\]
A.3 Proof of Theorem 2 and Lemma 1

Theorem 2. Consider a reaction network and fix $T$. Let $E, \varphi, i, j, I, J$ be as in Proposition 4. Assume that

\[ \text{itemsep=0pt} \]

- the sign of $\frac{1}{\varphi'(z)} \det(J_{F_{T,j}}(\varphi(z)))_{J,I}$ is independent of $z \in E$ and is nonzero, and
- the first $s-1$ Hurwitz determinants of $q_{s}(\lambda)$ are positive for all positive steady states $x^*$. If $z_{1} < \cdots < z_{t}$ are the positive solutions to the equation $F_{T,j}(\varphi(z)) = 0$, and all have multiplicity one, then either $\varphi(z_{1}), \varphi(z_{3}), \ldots$ are exponentially stable and $\varphi(z_{2}), \varphi(z_{4}), \ldots$ are unstable, or the other way around. Specifically, $\varphi(z_{1})$ is exponentially stable if

\[
\frac{(-1)^{s+t+j}}{\varphi'(z_{1})} (F_{T,j} \circ \varphi)'(z_{1}) \det(J_{F_{T,j}}(\varphi(z_{1})))_{J,I} > 0,
\]

and unstable if it is negative.

Proof. By the second hypothesis, the first $s-1$ Hurwitz determinants are positive, so the stability only depends on the sign of the last Hurwitz determinant, which as discussed in the main text, agrees with the sign of $(-1)^{s} \det(J_{F_{T,j}}(\varphi(z)))_{J,I}$. According to Proposition 4 it further coincides with the sign of $\frac{(-1)^{s+t+j}}{\varphi'(z)} (F_{T,j} \circ \varphi)'(z) \det(J_{F_{T,j}}(\varphi(z)))_{J,I}$. Since $\det(J_{F_{T,j}}(\varphi(z)))_{J,I}$ has a constant sign for every $z \in E$, the sign of the last Hurwitz determinant changes when the sign of $(F_{T,j} \circ \varphi)'(z)$ does, and this is the derivative of a univariate differentiable function whose real positive roots $z_{1} < \cdots < z_{t}$ have multiplicity one and are ordered in an increasing way. Given that $(F_{T,j} \circ \varphi)$ is differentiable, the sign of its derivative evaluated at consecutive roots alternates, that is $(F_{T,j} \circ \varphi)(z_{1}) > 0, (F_{T,j} \circ \varphi)(z_{3}) > 0, \ldots$ and $(F_{T,j} \circ \varphi)(z_{2}) < 0, (F_{T,j} \circ \varphi)(z_{4}) < 0, \ldots$ or the other way around.

In our setting this means that, once the sign of $(F_{T,j} \circ \varphi)(z_{k})$ is multiplied by $(-1)^{s+t+j}$ and by the sign of $\frac{1}{\varphi'(z)} \det(J_{F_{T,j}}(\varphi(z)))_{J,I}$, either $\varphi(z_{1}), \varphi(z_{3}), \ldots$ are exponentially stable and $\varphi(z_{2}), \varphi(z_{4}), \ldots$ are unstable, or the other way around. In particular, if the sign of $(F_{T,j} \circ \varphi)(z_{1})$ times the sign of $(-1)^{s+t+j} \frac{1}{\varphi'(z)} \det(J_{F_{T,j}}(\varphi(z)))_{J,I}$ is positive, then $\varphi(z_{1})$ is exponentially stable.

Lemma 1. Under the assumptions of Theorem 2, assume $(F_{T,j} \circ \varphi)(z) = \frac{a(z)}{b(z)}$ is a rational function in $z$ such that $b(z)$ is positive in $E$. Then the zeros of $(F_{T,j} \circ \varphi)(z)$ agree with the roots of $a(z)$ and the sign of $(F_{T,j} \circ \varphi)'(z^*)$, and $a'(z^*)$ agree for all $z^* \in E$ such that $a(z^*) = 0$.

Proof. The first part is straightforward, since the denominator of $(F_{T,j} \circ \varphi)(z)$ does not vanish in $E$. For the second part, we have $F_{T,j}(\varphi(z^*)) = \frac{a'(z^*)|b(z^*)|}{b(z^*)} = \frac{a'(z^*)}{b(z^*)}$, and the conclusion follows from the fact that $b(z^*) > 0$.

B Other algebraic criteria for stability

In this section we discuss two other criteria to decide the stability of the steady states.

Similarly to the Hurwitz criterion, the Liénard-Chipart criterion in [13], determines whether all the roots of a polynomial have negative real part, and requires a smaller amount of computations than the Hurwitz criterion. Before introducing the criterion, we need some ingredients.

Definition 1. • The Bezout matrix of two polynomials $h(x) = h_{n}x^{n} + h_{n-1}x^{n-1} + \cdots + h_{1}x + h_{0}$ and $g(x) = g_{m}x^{m} + g_{m-1}x^{m-1} + \cdots + g_{1}x + g_{0}$ with $n \geq m$, denoted by $B_{h,g}$, is defined as the representation matrix of the bilinear form given by

\[
B(h, g; x, y) = \frac{h(x)g(y) - h(y)g(x)}{x - y} = \sum_{i,j=0}^{n-1} b_{i+k}x^{k},
\]

that is, $B_{h,g} := (b_{ik})$. This is a symmetric matrix of size $n \times n$.  


A square matrix $A \in \mathbb{R}^{n \times n}$ is called a P-matrix if all its principals minors are positive, that is, $\det(A_{I,I}) > 0$ for every subset $I \subseteq \{1, \ldots, n\}$. If $A$ is symmetric, this is equivalent to $A$ being positive definite.

We now present the first additional stability criterion.

**Criterion 2** (Liénard-Chipart). All the roots of a polynomial $p(x) = x^n + p_{n-1}x^{n-1} + \ldots + p_1x + p_0$ with $p_i \in \mathbb{R}$ and $p_0 \neq 0$ have negative real part if and only if, after writing $p(x) = h(x^2) + xg(x^2)$, the Bezout matrix $B_{h,g}$ of $h$ and $g$ is positive definite and $p_i > 0$ for $i = 1, \ldots, s$.

In this criterion the polynomials $h$ and $g$ are associated with the even and odd parts of $p$ respectively. Note that the degrees of $h$ and $g$ are at most $\lfloor \frac{s}{2} \rfloor$, therefore the size of $B_{h,g}$ is $[\frac{s}{2}]$. Additionally, since $B_{h,g}$ is symmetric, $B_{h,g}$ is positive definite if and only if it is a P-matrix.

Unlike the Hurwitz criterion, Liénard-Chipart does not give a result regarding instability. If $B_{h,g}$ is not a P-matrix, it is not possible to determine whether the eigenvalues that do not have negative real part, have positive or zero real part.

In order to apply the criterion to the running example in the main text (Eq. (4)), we write $q_z(\lambda)$ as $h(\lambda^2) + \lambda g(\lambda^2)$, with

$$h(\lambda) = \lambda + \kappa_1 \kappa_2 x_2 + \kappa_2 \kappa_3 x_3 + \kappa_3 \kappa_1$$

and

$$g(\lambda) = \kappa_2 x_2 + \kappa_2 x_3 + \kappa_1 + \kappa_3.$$ 

The Bezout matrix is then $B_{h,g} = (x_2 + x_3) \kappa_2 + \kappa_1 + \kappa_3$, which is clearly a P-matrix. By the Liénard-Chipart criterion, we conclude that all the roots of $q_z(\lambda)$ have negative real part, recovering thereby that the only positive steady state in each stoichiometric compatibility class is exponentially stable.

The Liénard-Chipart criterion is the most efficient if we consider the amount of determinants that have to be computed to reach a decision. Given that the Bezout matrix is symmetric, to check that is positive definite, it is only necessary to compute the principal minors and check whether they are positive. Thus the required amount of determinants is at most $\sum_{\kappa=0}^{\lfloor \frac{s}{2} \rfloor} \binom{\lfloor \frac{s}{2} \rfloor}{\kappa}$. Although this criteria computes the smallest amount of determinants, for some examples, the entries of the Bezout matrix are larger than the entries of the Jacobian. In those cases the memory of a regular PC is still not enough to store the computations.

We conclude the list of stability criteria of algebraic nature with one more criterion, which does not rely on the computation of the characteristic polynomial. For a square matrix $A$, we say that $A$ is sign symmetric if $\det(A_{I,J}) \det(A_{J,I}) \geq 0$ for every $I, J \subset \{1, \ldots, n\}$ with the same cardinality.

**Criterion 3** (P-matrices that are sign symmetric). If a square matrix $A$ is both a P-matrix and sign symmetric, then all its eigenvalues have positive real part.

With this criterion, proved in [3], if $-A$ is a P-matrix and sign-symmetric, then all its eigenvalues have negative real part. For reaction networks, we apply the criterion to the matrix $-Q_x$. In the running example in the main text, we compute the minors of size 1 and 2 of $A = -Q_x$. The minors of size 1 are the entries of the matrix, which are all positive. The only minor of size 2 is

$$\det(A) = \kappa_1 \kappa_2 x_2 + \kappa_2 \kappa_3 x_3 + \kappa_1 \kappa_3.$$

All minors are polynomials that are positive for all $x \in \mathbb{R}^4_{>0}$ and positive $\kappa$. Therefore $-Q_x$ is a P-matrix and sign symmetric. Hence, with this new criterion we recover the conclusion that the only positive steady state is exponentially stable.

While the Hurwitz and Liénard-Chipart criteria are applied to the characteristic polynomial of $LJ_0(x^*)R_0$, which is independent of the choice of $R_0$, Criterion 3 is applied directly to the matrix $-LJ_0(x^*)R_0$. By Proposition 3 two different choices of $R_0$ give rise to two similar matrices. However, the properties of being P-matrix and sign symmetric are not preserved on similar matrices. As a small example consider

$$A = \begin{pmatrix} 2 & 1 \\ 3 & 4 \end{pmatrix}$$

and

$$B = \begin{pmatrix} -1 & -4 \\ 3 & 7 \end{pmatrix};$$
We rename the species as follows:

We consider the following network with mass-action kinetics

C.1 Hybrid histidine kinase

computations, you may request the Maple file to the authors.

In this section we provide extra details on the examples of the main text. For more details on the

C Examples

of information given that the entries of the Jacobian are typically polynomials.

system is

The concentration of the species

whether a matrix is a P-matrix and sign-symmetric requires the largest amount of operations. In this

case, all \( \sum_{i=1}^4 (s_i)^2 \) minors of the matrix must be computed, which requires the storage of a large amount

of information given that the entries of the Jacobian are typically polynomials.

C Examples

In this section we provide extra details on the examples of the main text. For more details on the

computations, you may request the Maple file to the authors.

C.1 Hybrid histidine kinase

We consider the following network with mass-action kinetics

\[
\begin{align*}
\text{HK}_0 & \xrightarrow{k_1} \text{HK}_p \quad \text{HK}_p & \xrightarrow{k_2} \text{HK}_0 \\
\text{HK}_p & \xrightarrow{k_3} \text{HK}_p \quad \text{HK}_p & \xrightarrow{k_4} \text{HK}_0 + \text{Htp}_p \\
\text{Htp}_p & \xrightarrow{k_5} \text{Htp} \quad \text{Htp} & \xrightarrow{k_6} \text{Htp} \\
\end{align*}
\]

We rename the species as follows: \( X_1 = \text{HK}_0, \ X_2 = \text{HK}_p, \ X_3 = \text{HK}_0, \ X_4 = \text{HK}_p, \ X_5 = \text{Htp} \) and \( X_6 = \text{Htp}_p \).

The conservation laws of the system are

\[
T_1 = x_1 + x_2 + x_3 + x_4 \\
T_2 = x_5 + x_6.
\]

Hence

\[
F_T(x) = \begin{pmatrix} x_1 + x_2 + x_3 + x_4 - T_1 \\
k_5x_1x_5 + k_4x_1 - k_2x_2 \\
-k_4x_1x_5 + k_2x_2 - k_3x_3 \\
-k_5x_4x_5 + k_3x_3 \\
x_5 + x_6 - T_2 \\
k_4x_3x_5 + k_5x_4x_5 - k_6x_6 \end{pmatrix}.
\]

Here \( s = 4 \). The existence of three positive steady states for this network was established in [31]. We compute \( q_s(\lambda) \) and the Hurwitz determinants in Maple and obtain that all but the last are polynomials

in \( x \) and \( \kappa \) with positive coefficients, hence positive when evaluated at a positive steady state.

We proceed to decide whether Theorem 2 applies. In [31], it was shown that the assumptions of

Proposition 4 hold with \( i = j = 5, \phi_5(z) = z \) and \( F_{T,5} \) corresponds to the conservation law with \( T_1 \). That is, the solutions to the four steady state equations together with the conservation law associated with \( T_1 \) can be parametrized by a function \( \varphi \) that only depends on \( z = x_5 \) and whose domain is \( \mathcal{E} = \mathbb{R}_{>0} \). The

denominator of \( (F_{T,5} \circ \varphi)(z) \) is positive and its numerator is a polynomial of degree 3 in \( z \), which can have 1, 2 or 3 positive roots, depending on the choice of parameters. Additionally, \( \det(J_{F_T(x)}(\varphi(x_1)))_{i,j} \) is a rational function with all coefficients positive. Thus, we are in the situation of Theorem 2 and can employ Lemma 1. Since the independent term of the numerator of \( (F_{T,5} \circ \varphi)(z) \) is negative, its first root has positive derivative. Further, the sign of \( \frac{(-1)^{j+j} \varphi_j(z)}{\varphi_j'(z_1)} \) is \( (-1)^{4+5+5} = +1 \), and hence, the sign of

\[
\frac{(-1)^{j+j} \varphi_j'(z_1)}{\varphi_j'(z_1)} \det(J_{F_T(x)}(\varphi(z_1)))_{i,j}
\]

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is positive as well. We conclude, using Theorem 2, that whenever the network has three positive steady states coming from the roots $z_1 < z_2 < z_3$ of $(F_{T,S} \circ \varphi)(z)$, then the steady states $\varphi(z_1)$ and $\varphi(z_2)$ are exponentially stable and the steady state $\varphi(z_2)$ is unstable. We have shown that this network displays bistability whenever there are three positive steady states.

C.2 Two substrate enzyme catalysis

We consider now the following network with mass-action kinetics

$$E + S_1 \xrightarrow{k_1 \kappa_1} ES_1 \quad E + S_2 \xrightarrow{k_2 \kappa_2} ES_2 \quad S_1 + ES_2 \xrightarrow{\kappa_5 \kappa_6} ES_1 S_2 \quad ES_1 S_2 \xrightarrow{\kappa_7 \kappa_8} E + P \quad S_1 + ES_2 \xrightarrow{\kappa_9 \kappa_{10}} ES_1 S_2.$$  

This network consists of an enzyme $E$ that binds two substrates, $S_1$ and $S_2$, in order to catalyze the reversible conversion to the product $P$. The binding is unordered. It was proven in [10] that this network has a unique steady state in each stoichiometric compatibility class for every set of reaction rate constants. We now prove that this steady state is exponentially stable. First, denote the species as $x_1 = E$, $x_2 = S_1$, $x_3 = ES_1$, $x_4 = S_2$, $x_5 = ES_2$, $x_6 = ES_1 S_2$, and $x_7 = P$. The concentration of species $x_i$ is denoted by $x_i$. With this notation the ODE system is

$$\begin{align*}
\frac{dx_1}{dt} &= -k_1 x_1^2 - k_3 x_1 x_4 - k_{10} x_1 x_7 + k_2 x_3 + k_4 x_5 + k_9 x_6,
\frac{dx_2}{dt} &= -k_2 x_2 - k_7 x_3 + k_5 x_4 + k_7 x_6,
\frac{dx_3}{dt} &= k_1 x_1 x_2 - k_5 x_3 x_4 - k_2 x_3 + k_6 x_6,
\frac{dx_4}{dt} &= -k_3 x_1 x_4 - k_5 x_3 x_4 + k_4 x_5 + k_6 x_6,
\frac{dx_5}{dt} &= k_3 x_1 x_2 - k_5 x_3 x_4 - k_2 x_3 + k_6 x_6.
\end{align*}$$

and the conservation laws are

$$x_1 + x_5 + x_6 + x_7 = T_1, \quad x_2 + x_3 + x_6 + x_7 = T_2 \quad \text{and} \quad x_1 + x_5 + x_6 + x_7 = T_3.$$  

With this choice of conservation laws we have

$$F_T(x) = \begin{pmatrix}
    x_1 + x_5 + x_6 - T_1 \\
    x_2 + x_3 + x_6 + x_7 - T_2 \\
    k_1 x_1 x_2 - k_5 x_3 x_4 - k_2 x_3 + k_6 x_6 \\
    x_4 + x_5 + x_6 + x_7 - T_3 \\
    k_3 x_1 x_4 - k_5 x_3 x_4 - k_4 x_5 + k_7 x_6 \\
    k_5 x_3 x_4 + k_5 x_2 x_5 + k_{10} x_1 x_7 - k_6 x_6 - k_7 x_6 - k_9 x_6 \\
    -k_{10} x_1 x_7 + k_9 x_6
\end{pmatrix}.$$  

Here $s = 4$. We compute $q_s(\lambda)$ and the Hurwitz determinants in Maple, and find that all but the last have all coefficients positive, and thus are positive.

We find next a positive parametrization by solving the steady state equations in the variables $x_3, x_5, x_6, x_7$, following the methods proposed in [22, 10]:

$$\begin{align*}
x_3 &= \frac{x_1 x_2 (k_3 k_5 k_8 x_2 + k_3 k_8 k_4 x_4 + k_1 k_4 k_7)}{k_2 k_6 k_8 x_2 + k_4 k_5 k_7 x_4 + k_2 k_4 k_6 + k_2 k_4 k_7},
x_5 &= \frac{x_1 x_4 (k_1 k_4 k_7 x_2 + k_3 k_5 k_7 x_4 + k_2 k_5 k_6 + k_2 k_5 k_7)}{k_2 k_6 k_8 x_2 + k_4 k_5 k_7 x_4 + k_2 k_4 k_6 + k_2 k_4 k_7},
x_6 &= \frac{x_1 x_2 x_4 (k_1 k_5 k_8 x_2 + k_2 k_5 k_6 + k_1 k_5 k_8)}{k_2 k_6 k_8 x_2 + k_4 k_5 k_7 x_4 + k_2 k_4 k_6 + k_2 k_4 k_7},
x_7 &= \frac{x_2 x_4 (k_1 k_5 k_8 x_2 + k_2 k_5 k_6 + k_1 k_5 k_8)}{k_2 k_6 k_8 x_2 + k_4 k_5 k_7 x_4 + k_2 k_4 k_6 + k_2 k_4 k_7} k_{10}.
\end{align*}$$

After evaluation of the independent term of $q_s(\lambda)$ at the parametrization, $H_4$ becomes positive. We conclude that for any choice of reaction rate constants, the network for two substrate enzyme catalysis has exactly one positive steady state in each stoichiometric compatibility class, which is exponentially stable.

C.3 Gene transcription network

We consider now the following gene transcription network shown in the main text:
We denote the species as mass-action kinetics. It was proven in [10] that for each set of positive reaction rate constants there is a and denote their concentrations with lower case letters. Additionally, we are under the assumption of the conservation laws are

and the conservation laws are

These give rise to the function $F_T(x)$:

$$F_T(x) = \begin{pmatrix} x_1 + x_7 - T_1 \\ x_2 + x_5 - T_2 \\ -\kappa_5 x_2 x_3 + k_1 x_1 - k_3 x_3 + k_6 x_5 \\ -2k_7 x_4^2 + k_2 x_2 - k_4 x_4 + 2k_8 x_6 \\ k_5 x_2 x_3 - k_6 x_5 \\ k_7 x_4^2 - k_9 x_1 x_6 - k_8 x_6 + k_10 x_7 \\ k_9 x_1 x_6 - k_10 x_7 \end{pmatrix}.$$  

Here $s = 5$. We find $q_s(\lambda)$ and compute the 5 Hurwitz determinants for $x \in \mathbb{R}^7_{>0}$. We find that $H_3, H_4, H_5$ have coefficients of both signs. We proceed to find a parametrization by solving the steady state equations in $x_1,\ldots,x_7$, which gives:

$$x_3 = \frac{k_1 x_1}{k_3}, \quad x_4 = \frac{k_2 x_2}{k_4}, \quad x_5 = \frac{k_1 k_5 x_1 x_2}{k_3 k_6}, \quad x_6 = \frac{k_2 k_7 x_4^2}{k_4 k_8}, \quad x_7 = \frac{k_2 k_7 k_9 x_6 x_2^2}{k_9 k_10}.$$  

After evaluating $H_3, H_4$ and $H_5$ in this parametrization, $H_3$ and $H_4$ become rational functions in $x_1, x_2$ and $\kappa$ with all coefficients positive. Hence they are positive as well.

This means that the stability of the steady state is determined by the sign of $H_5$. We check whether we can apply Theorem 2. By solving $F_T(x) = 0$ in $x_2,\ldots,x_7$ after removal of the conservation law with total amount $T_1$, we obtain

$$x_2 = \frac{k_3 k_6 T_2}{k_1 k_5 x_1 + k_3 k_6}, \quad x_3 = \frac{k_1 x_1}{k_3}, \quad x_4 = \frac{k_2 k_3 k_4 T_2}{(k_1 k_5 x_1 + k_3 k_6) k_4}, \quad x_5 = \frac{k_1 k_5 x_1 T_2}{k_1 k_5 x_1 + k_3 k_6}, \quad x_6 = \frac{k_2 k_9^2 k_7 T_2^2}{(k_1 k_5 x_1 + k_3 k_6)^2 k_2 k_8}, \quad x_7 = \frac{k_2 k_9^2 k_7 T_2^2}{(k_1 k_5 x_1 + k_3 k_6)^2 k_2 k_8}.$$  

These expressions define $\varphi$, with $z = x_1, \varphi_1(z) = z$ and $E = \mathbb{R}_{>0}$. By inserting these expressions into the conservation law with $T_1$, we conclude that the solutions of $F_T(x) = 0$ are in one to one correspondence with the zeroes of the function

$$(F_{T_1} \circ \varphi)(z) = \frac{1}{(k_1 k_5 z + k_3 k_6)^2 k_7 k_8 k_10 z^3 + (-T_1 k_1 k_5 + 2k_3 k_6) k_1 k_2 k_5 k_6 k_8 k_10 z^2 + (T_2 k_2 k_9 k_7 k_9 - 2T_1 k_1 k_5 k_2 k_5 k_6 k_8 k_10 + k_2 k_3 k_8 k_9 k_10)}{(k_1 k_5 x_1 + k_3 k_6)^2 k_2 k_8}.$$  

The numerator of this function has degree 3 in $z$, so using Lemma 1 the maximum number of positive steady states in each stoichiometric compatibility class is 3. We next verify that the first hypothesis in Theorem 2 is satisfied. Specifically, we compute

$$\frac{1}{\varphi_1^j(z)} \det(J_{F_T(\varphi(z))})_{j,l} = \det(J_{F_T(\varphi(z))})_{(2,\ldots,7),(2,\ldots,7)} = -(k_1 k_5 x_1 + k_3 k_6) k_4 k_8 k_10 < 0.$$  

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Since the sign of this polynomial is constant in \( E \), we apply Theorem 2. We have that the sign of
\[
\frac{(-1)^{s+i+1}}{\varphi_j'(z_1)} (F_{T,1} \circ \varphi)'(z_1) \det(J_{F_T}(\varphi(z_1))_{i,j})
\]
is positive since \( s + i + j = 7 \) and the sign of \((F_{T,1} \circ \varphi)'(z_1)\) is positive as the independent term of the numerator of \((F_{T,1} \circ \varphi)'(z_1)\) is negative. Therefore, the stability of the steady states alternate with \( z \) starting with an exponentially stable steady state. Specifically, if a stoichiometric compatibility class has one positive steady state, then it is exponentially stable. If it has three positive steady states, then two of them are exponentially stable and the other one is unstable. Bistability is guaranteed whenever the network has three positive steady states.

C.4 Monostationary networks from Fig. 3 in the main text

Networks (1) to (4) are straightforward to analyze, since all coefficients of the \( x \) and \( \kappa \) are positive; hence the Hurwitz determinants are positive for all \( x \in \mathbb{R}^\geq_0 \) and \( \kappa \in \mathbb{R}^\geq_0 \).

For networks (5) and (6) the computation was interrupted as it took long. In both networks \( s = 6 \), and the first four Hurwitz determinants could be computed. These determinants are polynomials in \( \kappa \) and \( x \) with positive coefficients, thus they are positive for every positive steady state. In order to compute \( H_5 \), we rely on an identity that holds for the Hurwitz determinants of a generic polynomial of degree 6. Namely, for a generic polynomial \( h(t) = a_0 t^6 + a_3 t^5 + a_4 t^4 + a_5 t^3 + a_2 t^2 + a_1 t + a_0 \), the fifth Hurwitz determinant can be written in terms of the previous ones as follows
\[
H_5 = a_1 H_4 + a_0(-a_0 a_3^2 + a_1 a_5 H_2 - a_3 H_3).
\]
With this identity, we analyze the sign of the coefficients of \( H_5 \) by studying separately the coefficients of \( A = a_0(-a_0 a_3^2 + a_1 a_5 H_2 - a_3 H_3) \) and \( B = a_1 H_4 \) after substituting \( a_i \) for the coefficient of \( \lambda^i \) in \( g_\mu(\lambda) \). First note that the coefficients of \( B \) are positive because both \( a_1 \) and \( H_4 \) are polynomials with positive coefficients. Now, to study the sign of \( A \) we identify each term \( \pi_a x^\alpha \) with a tuple of the form \((\pi_a, \alpha)\), that is, each term corresponds to a tuple whose first entry is its coefficient and the other entries correspond to the powers of the variables \( x_1, \ldots, x_{11} \) respectively. With this identification it is possible to store the polynomial using less memory and the sign of each coefficient can be explored by studying the first entry of each tuple. Note that each \( \pi_a \) is a polynomial in the rate constants \( \kappa \); furthermore, after analyzing their sign we found that these polynomials have negative coefficients. This implies that if \( H_5 \) has terms with negative coefficients, they come from monomials in \( A \). We use this to find the coefficients of \( H_5 \) by adding only monomials of \( B \) that have the same exponents to monomials in \( A \). In networks (5) and (6) this meant that we were analyzing only 24196 and 27982 coefficients instead of 37319 and 36970 coefficients respectively. After these computations we found that \( H_5 \) is a polynomial with positive coefficients. Therefore, positive for every positive steady state.

Knowing this about \( H_5 \), we also conclude that \( H_6 = a_0 H_5 \) is a polynomial with positive coefficients and the unique steady state in each stoichiometric compatibility class is exponentially stable for every set of parameters. Note that in these computations we do not need to evaluate at a positive parametrization, meaning all Hurwitz determinants are positive for arbitrary positive \( \kappa \) and \( x \).

C.5 Multistationary networks from Fig. 4 in the main text

We consider now the networks in Fig. 4 in the main text, which all are known to be multistationary. We sketch here why the procedure fails for each network, and how it applies to the reduced network.

\textbf{Network (a).} This network is the combination of two one-site modification cycles where the same kinase \( E \) activates the phosphorylation process and two different phosphates \( F_1 \) and \( F_2 \) catalyze the dephosphorylation process:
\[
S_0 + E \xrightarrow{\kappa_1 \ \ k_2} S_0 E \xrightarrow{\kappa_3} S_1 + E \quad S_1 + E \xrightarrow{\kappa_4 \ \ k_5} S_1 E \xrightarrow{\kappa_6} S_2 + E
\]
\[
S_1 + F_1 \xrightarrow{\kappa_7 \ \ k_8} S_1 F_1 \xrightarrow{\kappa_9} S_0 + F_1 \quad S_2 + F_2 \xrightarrow{\kappa_{10} \ \ k_{11}} S_2 F_2 \xrightarrow{\kappa_{12}} S_1 + F_2.
\]
The species are renamed as $S_0 = X_1, S_1 = X_2, S_2 = X_3, E = X_4, F_1 = X_5, F_2 = X_6$. $S_0E = X_7, S_1E = X_8, S_1F_1 = X_9, S_2F_2 = X_{10}$ and their concentrations are denoted in lower case letters.

Since the polynomial $q_4(\lambda)$ has degree 6, it is necessary to compute 6 Hurwitz determinants. These determinants were computed and their signs were analyzed up to $H_4$, and they have positive coefficients. However, the analysis of the sign of $H_5$ was interrupted as it was taking a long time to finish and it was not possible to store the polynomial in the expanded format in a regular PC. To compute and study this determinant more effectively, we use a monomial positive parametrization $\phi$ of the steady state variety, given by

\[ x_1 = \frac{(\kappa_2 + \kappa_3)(\kappa_5 + \kappa_6)\kappa_7\kappa_9\kappa_{10}\kappa_{12}}{\kappa_1\kappa_3\kappa_4\kappa_6(\kappa_8 + \kappa_9)(\kappa_{11} + \kappa_{12})} x_3 x_5 x_6 \]
\[ x_2 = \frac{(\kappa_5 + \kappa_6)\kappa_{10}\kappa_{12}}{\kappa_4\kappa_6(\kappa_{11} + \kappa_{12})} x_4 \]
\[ x_7 = \frac{(\kappa_5 + \kappa_6)\kappa_7\kappa_9\kappa_{10}\kappa_{12}}{\kappa_3\kappa_4\kappa_6(\kappa_8 + \kappa_9)(\kappa_{11} + \kappa_{12})} x_3 x_5 x_6 \]
\[ x_8 = \frac{\kappa_{10}\kappa_{12}}{\kappa_6(\kappa_{11} + \kappa_{12})} x_3 x_6 \]
\[ x_9 = \frac{(\kappa_5 + \kappa_6)\kappa_7\kappa_{10}\kappa_{12}}{\kappa_4\kappa_6(\kappa_8 + \kappa_9)(\kappa_{11} + \kappa_{12})} x_3 x_5 x_6 \]
\[ x_{10} = \frac{\kappa_{10}}{\kappa_{11} + \kappa_{12}} x_3 x_6. \]

This parametrization, written with the notation of the manuscript, corresponds to

\[ \alpha = \left( \frac{(\kappa_2 + \kappa_3)(\kappa_5 + \kappa_6)\kappa_7\kappa_9\kappa_{10}\kappa_{12}}{\kappa_1\kappa_3\kappa_4\kappa_6(\kappa_8 + \kappa_9)(\kappa_{11} + \kappa_{12})}, \frac{(\kappa_5 + \kappa_6)\kappa_{10}\kappa_{12}}{\kappa_4\kappa_6(\kappa_{11} + \kappa_{12})}, \frac{(\kappa_5 + \kappa_6)\kappa_7\kappa_9\kappa_{10}\kappa_{12}}{\kappa_3\kappa_4\kappa_6(\kappa_8 + \kappa_9)(\kappa_{11} + \kappa_{12})}, \frac{\kappa_{10}}{\kappa_{11} + \kappa_{12}} \right), \]
\[ B = \begin{bmatrix} 1 & 1 & 1 & 0 & 0 & 0 & 1 & 1 & 1 & 1 \\ -2 & -1 & 0 & 1 & 0 & 0 & -1 & 0 & -1 & 0 \\ 1 & 0 & 0 & 0 & 1 & 0 & 1 & 0 & 1 & 0 \\ 1 & 1 & 0 & 0 & 0 & 1 & 1 & 1 & 1 & 1 \end{bmatrix} \quad \text{and} \quad \xi = (x_3, x_4, x_5, x_6). \]

Using this parametrization and the identification of the monomials with tuples, it was possible to compute $H_5(\phi)$. However, the sign of this function remains unclear since it has coefficients with different signs.

We consider next the reduced network obtained by first removing all the reverse reactions and then the intermediates $S_1F_1$ and $S_2F_2$. When removing these intermediates the reactions $S_1 + F_1 \longrightarrow S_1 F_1 \longrightarrow S_0 + F_1$ and $S_2 + F_2 \longrightarrow S_2 F_2 \longrightarrow S_1 + F_2$ become $S_1 + F_1 \longrightarrow S_0 + F_1$ and $S_2 + F_2 \longrightarrow S_1 + F_2$ respectively. The reduced network is

\[ S_0 + E \stackrel{\tau_1}{\longrightarrow} S_0 E \stackrel{\tau_2}{\longrightarrow} S_1 + E \]
\[ S_1 + F_1 \stackrel{\tau_3}{\longrightarrow} S_0 + F_1 \]
\[ S_1 + E \stackrel{\tau_3}{\longrightarrow} S_1 E \stackrel{\tau_3}{\longrightarrow} S_2 + E \]
\[ S_1 + F_1 \stackrel{\tau_3}{\longrightarrow} S_2 + F_2 \]
\[ S_1 + F_1 \stackrel{\tau_3}{\longrightarrow} S_1 \quad \text{and} \quad \tau_5. \]

The species are now renamed as $S_0 = X_1, S_1 = X_2, S_2 = X_3, E = X_4, F_1 = X_5, F_2 = X_6$. Their concentrations are denoted in lower case letters.

The polynomial $q_4(\lambda)$ associated with this network has degree 4 and, when computing the Hurwitz determinants we have that $H_1, H_2$ and $H_3$ are positive. However the sign of $H_4$ is unclear even after evaluating in a positive parametrization of the steady state variety $\phi$. In this situation we explore the possibility of applying Theorem 2 to deduce bistability.

The conservation laws of the system are

\[ x_1 + x_2 + x_3 + x_7 + x_8 = T_1, \quad x_4 + x_7 + x_8 = T_2, \quad x_5 = T_3 \quad \text{and} \quad x_6 = T_4. \]

Taking the indices $i_1, i_2, i_3, i_4$ as $1, 4, 5, 6$ respectively, we construct $F_\ell$ as in equation [3] in the manuscript. The solutions of $F_{T,\ell} = 0$ for $\ell = 2, 3, 4, 5, 6$ are written in terms of $z = x_2$ as

\[ \varphi(z) = \left( \frac{\tau_7 \tau_3 (\tau_3 z + \tau_4) z}{\tau_1 \tau_4 (\tau_2 T_2 - \tau_5 T_3 z) z}, \frac{\tau_7 \tau_4 (\tau_2 T_2 - \tau_5 T_3 z)}{\tau_2 \tau_6 \tau_4 (\tau_3 z + \tau_4)} , \frac{\tau_4 (\tau_2 T_2 - \tau_5 T_3 z)}{\tau_2 (\tau_3 z + \tau_4)} , \frac{\tau_3 (\tau_2 T_2 - \tau_5 T_3 z) z}{\tau_2 (\tau_3 z + \tau_4)} , \frac{\tau_3 (\tau_2 T_2 - \tau_5 T_3 z) z}{\tau_2 (\tau_3 z + \tau_4)} , \frac{\tau_3 (\tau_2 T_2 - \tau_5 T_3 z) z}{\tau_2 (\tau_3 z + \tau_4)} , \frac{\tau_3 (\tau_2 T_2 - \tau_5 T_3 z) z}{\tau_2 (\tau_3 z + \tau_4)} \right), \]

for every $z \in \mathcal{E}$, where $\mathcal{E} = \left( 0, \frac{T_2 T_3}{T_3 T_7} \right)$. 


Note that $\varphi_2(z) = z$ and $\varphi'_2(z) = 1 \neq 0$. This means that the positive steady states in the stoichiometric compatibility class defined by $T$ are in one to one correspondence with the positive roots of $F_{T,1}(\varphi(z))$ in $E$. This rational function, presented below, has as numerator a polynomial of degree 3.

$$F_{T,1}(\varphi(z)) = \frac{1}{T_4T_5T_6T_7T_8T_9T_{10}} \left( -T_7T_8T_9T_{10}T_1T_2T_3T_4T_5T_6T_7T_8T_9T_{10} + T_5T_6T_7T_8T_9T_{10}T_1T_2T_3T_4T_5T_6T_7T_8T_9T_{10} ight) z^3$$

$$- T_7T_8T_9T_{10}T_1T_2T_3T_4T_5T_6T_7T_8T_9T_{10} + T_5T_6T_7T_8T_9T_{10}T_1T_2T_3T_4T_5T_6T_7T_8T_9T_{10}$$

$$- T_7T_8T_9T_{10}T_1T_2T_3T_4T_5T_6T_7T_8T_9T_{10}$$

$$- T_7T_8T_9T_{10}T_1T_2T_3T_4T_5T_6T_7T_8T_9T_{10}$$

We have already shown that the second hypothesis of Theorem 2 holds. For the first hypothesis, a straightforward computation shows that

$$\det(J_{F_T}(\varphi(z))) = \tau_1T_4T_5T_6T_7T_8T_9T_{10}$$

which is negative for every $z \in E$. We further have $s = 4, i = 2, j = 1$, and the independent term of the numerator of $F_{T,1}(\varphi(z))$ is negative, meaning that $(F_{T,j}(\varphi))(z_i) > 0$. This gives that the sign of

$$\frac{(-1)^{s+i+j}}{\varphi'_i(z_i)} (F_{T,j}(\varphi))(z_i) \det(J_{F_T}(\varphi(z)))$$

is $(-1)^{s+i+j}(1) = 1$ positive. Using Theorem 2, we conclude that for every set of parameters such that $F_{T,1}(\varphi(z))$ has three roots $z_1 < z_2 < z_3$ in $E$, the steady states $\varphi(z_1), \varphi(z_3)$ are exponentially stable and $\varphi(z_2)$ is unstable.

All that is left is to show that the reduced network admits three positive steady states in some stoichiometric compatibility class for some choice of $\tau$, or what is the same, that $F_{T,1}(\varphi(z))$ admits three roots in $E$. To this end, we apply the method from [10], which consists of finding values for the rate constants and concentration variables such that $\det(J_{F_T}(\phi))$, where $\phi$ is a parametrization of the steady states, is negative. We have

$$\phi(x_3, x_4, x_5, x_6) = \left( \frac{\tau_7 \tau_6 \tau_5 \tau_4 x_3^2 x_5^2 x_6}{\tau_3 x_4}, \frac{\tau_6 x_3 x_5 x_6}{\tau_3 x_4}, x_3, x_4, x_5, x_6, \frac{\tau_7 \tau_6 x_3 x_5 x_6}{\tau_4}, \frac{\tau_6 x_3 x_5}{\tau_4} \right)$$

and

$$\det(J_{F_T}(\phi)) = \tau_1 \tau_2 \tau_3 \tau_4^2 x_3^2 x_5^2 x_6^2 - \tau_1 \tau_4 \tau_5 \tau_6 x_3 x_5 x_6 x_6^2 + 2 \frac{\tau_2 \tau_5 \tau_6 x_3^2 x_5^2 x_6^2}{x_4} + \frac{\tau_4 x_4^2}{\tau_2}$$

$$+ \tau_1 \tau_2 \tau_3 \tau_6 x_4^2 x_6 + \tau_1 \tau_4 \tau_5 \tau_6 x_4^2 x_6 + \tau_1 \tau_2 \tau_3 \tau_6 x_2^2 + \tau_1 \tau_4 \tau_5 \tau_6 x_4 x_6 + \tau_2 \tau_4 \tau_5 \tau_6 x_5 x_6.$$

By setting $\tau_i = 1, i = 1, \ldots, 6$ and $x_3 = 100, x_4 = 10, x_5 = 10, x_6 = 1$, this determinant is $-280$, which is negative. This implies that the stoichiometric compatibility class containing $\phi(100, 10, 10, 1)$ has more than one positive steady state. Specifically, this class corresponds to $T_1 = 320, T_2 = 210, T_3 = 10, T_4 = 1$. Either by solving the steady state equations or finding the roots of $F_{T,1}(\varphi(z))$ for this choice of parameters, we confirm that the system has three positive steady states.

Therefore, the reduced network is bistable for all choice of parameter values for which there are three positive steady states, and the original network admits bistability in some region of the parameter space. **Network (B).** This network is the combination of two one-site modification cycles in a cascade, where the same phosphatase $F$ acts in both layers.

$$S_0 + E \xrightarrow{\epsilon_1} S_0 E \xrightarrow{\epsilon_3} S_1 + E \quad S_1 + F \xrightarrow{\epsilon_4} S_1 F \xrightarrow{\epsilon_6} S_0 + F$$

$$P_0 + S_1 \xrightarrow{\epsilon_{10}} P_0 S_1 \xrightarrow{\epsilon_{12}} P_1 + S_1 \quad P_1 + F \xrightarrow{\epsilon_{11}} P_1 F \xrightarrow{\epsilon_{13}} P_0 + F.$$
parametrization \( \phi \). We use the identification of monomials with tuples, to compute and analyze \( H_4(\phi) \) more efficiently. With the notation of the manuscript \( \phi \) corresponds to

\[
\alpha = \left( \begin{array}{c}
\frac{k_4k_6k_{10}k_12(k_2 + k_3)(k_8 + k_9)}{k_1k_3k_7k_9(k_5k_{11} + k_9k_{12})}, 1, 1, (k_8 + k_9)k_{10}k_{12}, 1, 1,
\frac{k_4k_6k_{10}k_12(k_8 + k_9)}{k_3k_7k_9(k_5k_{11} + k_9k_{12})(k_7k_9 k_5k_{11} + k_9k_{12})} , k_5(k_11 + k_{12}) \end{array} \right) \left( \begin{array}{c}
k_11 + k_{12}
\end{array} \right)
\]

\[
B = \left[ \begin{array}{cccccc}
2 & 1 & 0 & 2 & 0 & 0 \\
-1 & 0 & 1 & 0 & 0 & 0 \\
1 & 0 & 0 & 1 & 0 & 1 \\
1 & 0 & 0 & 1 & 1 & 1
\end{array} \right] \quad \text{and} \quad \xi = (x_2, x_3, x_5, x_6).
\]

Using this identification it was possible to compute \( H_4(\phi) \). However, its sign was still unclear as we encountered both positive and negative coefficients.

We then proceed to reduce the network by removing all reverse reactions and the intermediates \( S_9E \) and \( S_1F \). That is, the reactions \( S_0 + E \rightarrow S_0E \rightarrow S_1 + E \) and \( S_1 + F \rightarrow S_1F \rightarrow S_0 + F \) are transformed into \( S_0 + E \rightarrow S_1 + E \) and \( S_1 + F \rightarrow S_0 + F \) respectively. We are left with the following reduced network

\[
S_0 + E \xrightarrow{\tau_1} S_1 + E \quad \quad S_1 + F \xrightarrow{\tau_7} S_0 + F
\]

\[
P_0 + S_1 \xrightarrow{\tau_3} P_0S_1 \xrightarrow{\tau_7} P_1 + S_1 \quad \quad P_1 + F \xrightarrow{\tau_7} P_1F \xrightarrow{\tau_0} P_0 + F.
\]

In this network we rename the species as \( E = X_1, F = X_2, S_0 = X_3, S_1 = X_4, P_0 = X_5, P_1 = X_6, P_0S_1 = X_9, P_1F = X_{10} \). Their concentrations are denoted with lower case letters. The polynomial \( g_4(\lambda) \) has degree 4 and, after computing the Hurwitz determinants, we have that \( H_1, H_2, H_3 \) are positive. Therefore, the stability of the positive steady states depends on the sign of \( H_4(\phi) \). In this situation we explore the possibility of applying Theorem 2 to ensure bistability.

The conservation laws of the system are

\[
x_1 = T_1, \quad x_5 + x_6 + x_9 + x_{10} = T_2, \quad x_3 + x_4 + x_9 = T_3, \quad x_2 + x_{10} = T_4.
\]

Taking the indices \( i_1 = 1, i_2 = 2, i_3 = 3, i_4 = 5 \) we define \( F_T \) as in equation [3] in the manuscript. Furthermore, the solutions of \( F_{T,\ell} = 0 \) for \( \ell \neq 5 \) can be positively parametrized in terms of \( z = x_6 \) as

\[
\varphi(z) = \left( T_1, \frac{\tau_6 T_4}{\tau_3 z + \tau_6}, \frac{T_4 T_2 T_6 ((T_3 \tau_4 - T_4 \tau_6) \tau_2 z + T_3 \tau_4 \tau_6)}{\tau_2 \tau_3 ((T_1 \tau_1 z + \tau_6)(T_1 \tau_1 z + \tau_6) + T_4 \tau_2 \tau_6)}, \frac{1}{\tau_2 \tau_3 ((T_1 \tau_1 z + \tau_6)(T_1 \tau_1 z + \tau_6) + T_4 \tau_2 \tau_6)} \right).
\]

for every \( z \in \mathcal{E} \), where \( \mathcal{E} = \mathbb{R}_{\geq 0} \) if \( T_3 \tau_4 - T_4 \tau_6 > 0 \) or \( \mathcal{E} = \left( 0, \frac{T_3 \tau_4 \tau_6}{\tau_2 (\tau_1 z + \tau_6)} \right) \) if \( T_3 \tau_4 - T_4 \tau_6 \leq 0 \). Note that the positive steady states in the stoichiometric compatibility class defined by \( T \) are in one to one correspondence with the zeros of \( F_{T,5}(\varphi(z)) \), below, contained in \( \mathcal{E} \).

The numerator of this univariate rational function has degree 3 and the denominator is positive in \( \mathcal{E} \). Additionally, \( \varphi_0(z) = z \) and \( \varphi_0'(z) = 1 \neq 0 \). With this parametrization where \( i = 6, j = 5 \), we have all the elements required in the statement of Theorem 2. We also know from the analysis of the Hurwitz determinants that the second hypothesis of Theorem 2 holds. It remains to see that the first hypothesis of the theorem holds, but this is verified with a simple computation: The polynomial

\[
\det(J_{F_T}(\varphi(z)), I, I) = -\tau_3 T_1 (T_3 \tau_4 - T_4 \tau_6) \tau_5 z + T_3 \tau_4 \tau_6)
\]
is negative for every $z \in \mathcal{E}$. Using Theorem 2 and the fact that the independent term of $F_{T,5}(\varphi(z))$ is negative, we conclude that for every set of parameters such that $F_{T,5}(\varphi(z))$ has 3 positive roots $z_1 < z_2 < z_3$ in $\mathcal{E}$, the sign of

$$
\frac{(-1)^{s+i+j}}{\varphi'(z_1)} (F_{T,j} \circ \varphi)'(z_1) \det(J_{F_T}(\varphi(z_1)))_{I,I}
$$

is $(-1)^{6+5+4}(1)(-1) = 1$ positive and thus $\varphi(z_1), \varphi(z_3)$ are exponentially stable and $\varphi(z_2)$ is unstable.

It remains to see that there is a set of parameters such that the network admits three positive steady states. To do so, we apply the method from [10] and find a set of parameters and a steady state such that $\det(J_{F_2}((\phi))) < 0$ given a parametrization $\phi$. We have

$$
\phi(x_2, x_3, x_5, x_6) = \left(\frac{\tau_2 \tau_5 x_2^2 x_6}{\tau_1 \tau_3 x_3 x_5}, x_2, x_3, \frac{\tau_5 x_2 x_6}{\tau_3 x_5}, \frac{\tau_5 x_2 x_6}{\tau_4}, \frac{\tau_5 x_2 x_6}{\tau_6}\right)
$$
and

$$
\det(J_{F_T}(\phi)) = \frac{1}{\tau_3 x_3 x_5^2} \left(\tau_2 \tau_5 x_2^2 \left(\tau_3 \tau_4 \tau_5 x_2 x_3 x_5 x_6 - \tau_3 \tau_4 \tau_5 x_2 x_3 x_5 x_6 + \tau_3 \tau_4 \tau_5 x_2 x_3 x_5 x_6 + \tau_3 \tau_5 x_2 x_3 x_5 x_6 + \tau_3 \tau_5 x_2 x_3 x_5 x_6 + \tau_3 \tau_5 x_2 x_3 x_5 x_6 + \tau_3 \tau_5 x_2 x_3 x_5 x_6 + \tau_3 \tau_5 x_2 x_3 x_5 x_6 + \tau_3 \tau_5 x_2 x_3 x_5 x_6 + \tau_3 \tau_5 x_2 x_3 x_5 x_6 \right) \right).
$$

Taking $\tau_i = 1$ for $i = 1, \ldots, 6$ and $x_2 = 1, x_3 = 10, x_5 = 20, x_6 = 10$ this determinant is -4500. This implies that the stoichiometric compatibility class containing $\phi(1, 10, 20, 10)$ has more than one positive steady state. The total amounts defining it are $T_1 = \frac{1}{20}, T_2 = 50, T_3 = \frac{1}{2}, T_4 = 11$. Using these parameters and solving either $F_T(x) = 0$ or finding the roots of $F_{T,5}(\varphi)$ we verify that this stoichiometric compatibility class has in fact three positive steady states.

We conclude that the reduced network is bistable for every set of parameters for which there are three positive steady states, and the original network admits bistability in some region of the parameter space.

**Network (c).** This network is the combination of two one-site modification cycles in a cascade, where the same kinase $E$ acts in both layers:

$S_0 + E \overset{k_{13}}{\underset{k_{12}}{\rightleftharpoons}} S_0E \overset{k_3}{\rightarrow} S_1 + E$

$S_1 + F_1 \overset{k_4}{\underset{k_5}{\rightleftharpoons}} S_1F_1 \overset{k_2}{\rightarrow} S_0 + F_1$

$P_0 + S_1 \overset{k_7}{\underset{k_8}{\rightleftharpoons}} P_0S_1 \overset{k_9}{\rightarrow} P_1 + S_1$

$P_0 + E \overset{k_{13}}{\underset{k_{14}}{\rightleftharpoons}} P_0E \overset{k_{15}}{\rightarrow} P_1 + E$

$P_1 + F_2 \overset{k_{10}}{\underset{k_{11}}{\rightleftharpoons}} P_1F_2 \overset{k_{12}}{\rightarrow} P_0 + F_2.$

The species are renamed as $S_0 = X_1, S_1 = X_2, P_0 = X_3, P_1 = X_4, E = X_5, F_1 = X_6, F_2 = X_7, S_0E = X_8, S_1F_1 = X_9, P_0S_1 = X_{10}, P_1F_2 = X_{11}, P_0E = X_{12}$ and their concentrations are denoted with lower case letters. For this network, the polynomial $q_c(\lambda)$ has degree 7 and the determinants $H_1, H_2, H_3$ are positive polynomials. However, the computations of the other determinants was not possible as there was not enough memory to store the computations in a regular PC. In this case, we could not parametrize the positive steady state variety by monomials; therefore, it is not possible to use the identification between monomials and tuples to analyze the sign of the remaining determinants.

We proceed directly to reduce the network by removing all the reverse reactions first, and then the intermediates $S_0E, P_0S_1$ and $P_1F_2$. That is, the reactions $S_0 + E \rightarrow S_0E \rightarrow S_1 + E, P_0 + S_1 \rightarrow P_0S_1 \rightarrow P_1 + S_1$ and $P_1 + F_2 \rightarrow P_1F_2 \rightarrow P_0 + F_2$ are transformed into $S_0 + E \rightarrow S_1 + E,$
$P_0 + S_1 \longrightarrow P_1 + S_1$ and $P_1 + F_2 \longrightarrow P_0 + F_2$ respectively. We are left with the following network

\[
\begin{align*}
S_0 & \xrightarrow{\tau_1} S_1 + E \\
S_1 + F_1 & \xrightarrow{\tau_2} S_1F_1 \xrightarrow{\tau_3} S_0 + F_1 \\
P_0 + S_1 & \xrightarrow{\tau_4} P_1 + S_1 \\
P_0 + E & \xrightarrow{\tau_5} P_0E \xrightarrow{\tau_7} P_1 + E \\
P_1 + F_2 & \xrightarrow{\tau_6} P_0 + F_2.
\end{align*}
\]

The species are renamed as $S_0 = X_1, S_1 = X_2, P_0 = X_3, P_1 = X_4, E = X_5, F_1 = X_6, F_2 = X_7, S_1F_1 = X_9, P_0E = X_{12}$ and their concentrations are denoted by lower case letters. The polynomial $q_5(\lambda)$ associated to this network has degree 4 and $H_1, H_2, H_3$ are positive after evaluating in the following positive parametrization $\phi$ of the steady state variety:

\[
\phi(x_1, x_2, x_3, x_6, x_7) = (x_1, x_2, x_3, x_6, x_7, \frac{2x_1(x_1 + \tau_2 x_2 x_6)}{\tau_3 x_3 x_1 x_7}, \frac{\tau_2 x_2 x_6}{\tau_1 x_3}, \frac{x_6, x_7, \tau_2 x_2 x_6, \tau_6 x_3}{\tau_3}, \frac{\tau_2 x_2 x_6}{\tau_1 x_7}).
\]

However, the sign of $H_4$ is unclear. We now explore the use of Theorem 2 to ensure bistability. The conservation laws of the system are

\[
x_1 + x_2 + x_3 = T_1, \quad x_3 + x_4 + x_{12} = T_2, \quad x_5 + x_{12} = T_3, \quad x_6 + x_9 = T_4, \quad x_7 = T_5.
\]

Taking $i_1 = 1, i_2 = 3, i_3 = 5, i_4 = 6, i_5 = 7$ we define $F_T(x)$ as in equation [3] of the manuscript. Additionally, the solutions of $F_{T,\ell}(x) = 0$ for $\ell \neq 6$ can be parametrized in terms of $z = x_3$ as

\[
\varphi(z) = \frac{b_1(z)\tau_3 + b_2(z)\tau_6}{(\tau_3 x_3 + T_3 x_1 + \tau_3 x_7 + \tau_3 x_1 x_7)\tau_6} \cdot \frac{z_1}{\tau_3 x_3 + \tau_3 x_7 + \tau_3 x_1 x_7} \cdot \frac{(\tau_3 x_3 + T_3 x_1 + \tau_3 x_7 + \tau_3 x_1 x_7)\tau_6}{\tau_3 x_3 + \tau_3 x_7 + \tau_3 x_1 x_7}.
\]

where $b_1(z) = (T_1 x_1 + T_3 x_3)\tau_6 - (T_2 x_2 + T_3 x_3)\tau_6 + T_1 x_4 + T_3 x_3 + T_4 x_1 x_7 + T_5 x_5 x_7)z - T_2 x_3 + T_5 x_5 x_7$,

\[
b_2(z) = -T_3 x_3 - T_3 x_3 + T_6 x_3 + T_3 x_3 + T_4 x_1 x_7 - T_5 x_5 x_7)z + T_2 x_3 + T_5 x_5 x_7.
\]

The parametrization is positive if and only if $b_1(z), b_2(z) > 0$. This happens for every $z \in \mathcal{E}$, where $\mathcal{E} = \{\beta_1, \beta_2\}$ with $\beta_1$ and $\beta_2$ the (only) positive roots of the polynomials $b_1$ and $b_2$ respectively.

The steady states in each stoichiometric compatibility class are in one to one correspondence with the roots of $F_{T,6}(\varphi(z))$ in $\mathcal{E}$. The numerator of this function is a polynomial of degree 5 and the denominator is positive in $\mathcal{E}$. Additionally, $\varphi_3(z) = z$ and $\varphi_4(z) = 0 \neq 1$. With this parametrization and taking $i = 3, j = 6, s = 4$, we have all the elements in the statement of Theorem 2. From the computations before, we know that the first three Hurwitz determinants are positive and thus, the second hypothesis of the theorem holds. We only need to verify the first hypothesis of the theorem, but this is done by computing the required determinant

\[
\det(J_F(\varphi(z)))_{i,j} = -\tau_2(\tau_3 \tau_5 \tau_6 + T_3 \tau_1 \tau_7 + \tau_3 \tau_7) b_2(z) / \tau_6 \tau_7 + \tau_7.
\]

which is negative for every $z \in \mathcal{E}$. We are then in the setting of Theorem 2. We need to decide the sign of $(F_{T,\ell} \circ \varphi)'(z_1)$ at the first root of the numerator of $F_{T,\ell} \circ \varphi$. Indirect evaluation of $F_{T,\ell} \circ \varphi$ at $\beta_1$, by isolating $T_2$ from $b_2(z) = 0$ and substitution into $F_{T,\ell} \circ \varphi$, shows that $F_{T,\ell} \circ \varphi$ is negative at $\beta_1$. Hence the derivative at $z_1$ is positive. We conclude that for every set of parameters such that $F_{T,6}(\varphi(z))$ has more than two positive roots $z_1, z_2, \ldots$, the sign of

\[
(-1)^{s+t+j}(\varphi_4'(z_1)(F_{T,\ell} \circ \varphi)'(z_1) \det(J_F(\varphi(z_1)))_{i,j})
\]

is $(-1)^{s+t+j}(1)(-1) = 1$ positive. Therefore, the steady states $\varphi(z_1), \varphi(z_3), \ldots$ are exponentially stable and $\varphi(z_2), \ldots$ are unstable.

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It remains to see that there is a set of parameters such that the stoichiometric compatibility class in fact contains more than two positive steady states. This is done with the method presented in [10], that relies on the sign of \( \det(J_F(\phi)) \), with \( \phi \) as above. According to the method, we have to find a set of parameters and a steady state such that \( \det(J_F(\phi)) \) is negative. The stoichiometric compatibility class containing these steady states, contains at least another one. In this network

\[
\det(J_F(\phi)) = -71^2 \cdot \tau_1 \cdot \tau_0 \cdot x_2 \cdot x_3 \cdot x_6 + \tau_1 \cdot \tau_2 \cdot \tau_3 \cdot \tau_4 \cdot x_7 \cdot x_6 \cdot x_7 + \tau_1 \cdot \tau_2 \cdot \tau_3 \cdot \tau_4 \cdot \tau_5 \cdot x_7 \cdot x_6 \cdot x_7 + \tau_1 \cdot \tau_2 \cdot \tau_3 \cdot \tau_4 \cdot \tau_5 \cdot \tau_6 \cdot x_7 \cdot x_6 \cdot x_7 + \tau_1 \cdot \tau_2 \cdot \tau_3 \cdot \tau_4 \cdot \tau_5 \cdot \tau_6 \cdot \tau_7 \cdot x_7 \cdot x_6 \cdot x_7 + \tau_1 \cdot \tau_2 \cdot \tau_3 \cdot \tau_4 \cdot \tau_5 \cdot \tau_6 \cdot \tau_7 \cdot \tau_8 \cdot x_7 \cdot x_6 \cdot x_7 + \tau_1 \cdot \tau_2 \cdot \tau_3 \cdot \tau_4 \cdot \tau_5 \cdot \tau_6 \cdot \tau_7 \cdot \tau_8 \cdot \tau_9 \cdot x_7 \cdot x_6 \cdot x_7 + \tau_1 \cdot \tau_2 \cdot \tau_3 \cdot \tau_4 \cdot \tau_5 \cdot \tau_6 \cdot \tau_7 \cdot \tau_8 \cdot \tau_9 \cdot \tau_10 \cdot x_7 \cdot x_6 \cdot x_7 + \tau_1 \cdot \tau_2 \cdot \tau_3 \cdot \tau_4 \cdot \tau_5 \cdot \tau_6 \cdot \tau_7 \cdot \tau_8 \cdot \tau_9 \cdot \tau_10 \cdot \tau_11 \cdot x_7 \cdot x_6 \cdot x_7 + \tau_1 \cdot \tau_2 \cdot \tau_3 \cdot \tau_4 \cdot \tau_5 \cdot \tau_6 \cdot \tau_7 \cdot \tau_8 \cdot \tau_9 \cdot \tau_10 \cdot \tau_11 \cdot \tau_12 \cdot x_7 \cdot x_6 \cdot x_7 + \tau_1 \cdot \tau_2 \cdot \tau_3 \cdot \tau_4 \cdot \tau_5 \cdot \tau_6 \cdot \tau_7 \cdot \tau_8 \cdot \tau_9 \cdot \tau_10 \cdot \tau_11 \cdot \tau_12 \cdot \tau_13 \cdot x_7 \cdot x_6 \cdot x_7 + \tau_1 \cdot \tau_2 \cdot \tau_3 \cdot \tau_4 \cdot \tau_5 \cdot \tau_6 \cdot \tau_7 \cdot \tau_8 \cdot \tau_9 \cdot \tau_10 \cdot \tau_11 \cdot \tau_12 \cdot \tau_13 \cdot \tau_14 \cdot x_7 \cdot x_6 \cdot x_7 + \tau_1 \cdot \tau_2 \cdot \tau_3 \cdot \tau_4 \cdot \tau_5 \cdot \tau_6 \cdot \tau_7 \cdot \tau_8 \cdot \tau_9 \cdot \tau_10 \cdot \tau_11 \cdot \tau_12 \cdot \tau_13 \cdot \tau_14 \cdot \tau_15 \cdot x_7 \cdot x_6 \cdot x_7 + \tau_1 \cdot \tau_2 \cdot \tau_3 \cdot \tau_4 \cdot \tau_5 \cdot \tau_6 \cdot \tau_7 \cdot \tau_8 \cdot \tau_9 \cdot \tau_10 \cdot \tau_11 \cdot \tau_12 \cdot \tau_13 \cdot \tau_14 \cdot \tau_15 \cdot \tau_16 \cdot x_7 \cdot x_6 \cdot x_7 \]
We then proceed to **reduce the network** by removing all the reverse reactions and the intermediates $S_1E, S_2F$ and $S_3F$. That is, the reactions $S_1 \rightarrow S_2 \rightarrow S_3 \rightarrow S_4 \rightarrow S_5 \rightarrow S_6$ and $S_1 \rightarrow S_2 \rightarrow S_3 \rightarrow S_4 \rightarrow S_5 \rightarrow S_6$ become $S_1 \rightarrow S_2 \rightarrow S_3 \rightarrow S_4 \rightarrow S_5 \rightarrow S_6$ respectively. The reduced network is

$$
S_0 + E \xrightarrow{r_7} ES_0 \xrightarrow{r_2} S_1 + E \xrightarrow{r_3} S_2 + E
S_2 + F \xrightarrow{r_4} S_1 + F \xrightarrow{r_6} S_0 + F.
$$

We rename the species as $E = X_1, F = X_2, S_0 = X_3, S_1 = X_4, S_2 = X_5, S_0E = X_6$ and their concentrations are denoted with lowercase letters. The polynomial $q_2(\lambda)$ has degree 3 and, after computing the Hurwitz determinants, it was possible to prove that $H_1$ and $H_2$ are polynomials with positive coefficients and, thus, sign for every positive equilibrium state. However, the sign of $H_3$ was unclear even after evaluating at the parametrization $\phi$ of the positive steady state variety below:

$$
\phi(x_1, x_2, x_3) = \left( x_1, x_2, x_3, \frac{r_1x_1x_3}{r_5x_2}, \frac{r_3x_1x_3}{r_7x_8x_9}, \frac{r_1x_1x_3}{r_2} \right).
$$

We now check if it is possible to apply Theorem 2 to deduce bistability. The conservation laws of the system are

$$
x_1 + x_6 = T_1, \quad x_2 = T_2 \quad \text{and} \quad x_3 + x_4 + x_5 + x_6 = T_3.
$$

Taking $i_1 = 1, i_2 = 2, i_3 = 3$ we define $F_T(x)$ as in equation [3] of the manuscript. The solutions of $F_{T,5}(x) = 0$ for $\ell = 2, \ldots, 6$ can be parametrized in terms of $z = x_1$ as

$$
\varphi(z) = \left( z, T_2, \frac{T_2^2T_3^2T_7T_5}{r_1T_2T_7T_5T_8^3 + T_2T_3T_7T_5T_8^2 + T_2^2T_3T_7T_5T_8}, \frac{T_2T_3T_7T_5}{r_1T_2T_7T_5T_8^3 + T_2T_3T_7T_5T_8^2 + T_2^2T_3T_7T_5T_8}, \frac{T_2T_3T_7T_5}{r_1T_2T_7T_5T_8^3 + T_2T_3T_7T_5T_8^2 + T_2^2T_3T_7T_5T_8} \right).
$$

This parametrization is positive for every $z \in E = \mathbb{R}_{>0}$ and the positive steady states in the stoichiometric compatibility class are in one to one correspondence with the positive roots of $F_{T,5}(\varphi(z))$. This is a rational function whose numerator is a polynomial of degree 3 and positive denominator:

$$
F_{T,5}(\varphi(z)) = \frac{1}{r_1T_2T_7T_5T_8^3 + T_2T_3T_7T_5T_8^2 + T_2^2T_3T_7T_5T_8} \left[ r_1T_2T_7T_5T_8^3 + (T_2^2T_3T_7T_5T_8^2 + T_2^3T_3T_7T_5T_8)z^2 + (-T_1T_2^2T_3T_7T_5T_8T_9^2 + T_1T_2T_3T_7T_5T_8T_9)z + T_1T_2T_3T_7T_5T_8T_9 \right].
$$

Therefore, there are at most three positive steady states in each stoichiometric compatibility class. Additionally $\varphi_1(z) = z$ and $\varphi_1'(z) = 1 \neq 0$. With this parametrization, where, $i = j = 1$ and $s = 3$, we have all the elements in the statement of Theorem 2. From the analysis of the Hurwitz determinants we know that the second hypothesis of the theorem holds. It remains to see whether the first hypothesis also holds. The determinant that we have to study is

$$
\det(J_F(\varphi(z)), J_I) = -T_2^2T_3T_7T_5z - r_1T_2T_7T_5z^2 - T_2T_3T_7T_5z^2 - T_2^2T_3T_7T_5z,
$$

which is negative for every $z \in \mathbb{R}_{>0}$. We are in the setting of Theorem 2 and we conclude that for every set of parameters such that $F_{T,5}(\varphi(z))$ has three positive roots $z_1 < z_2 < z_3$, the sign of $(F_{T,j} \circ \varphi)'(z_1)$ is positive as the independent term of $(F_{T,j} \circ \varphi)(z)$ is negative. Furthermore, the sign of

$$
(\varphi_1'(z_1))^{-1} (F_{T,j} \circ \varphi)'(z_1) \det(J_F(\varphi(z)), J_I)
$$

is equal to $(-1)^{1+s}(-1) = 1$, which is positive. This implies that the steady states $\varphi(z_1), \varphi(z_3)$ are exponentially stable and $\varphi(z_2)$ is unstable.

We now verify that there exist a set of parameters such that the network has three positive steady states. In order to do so, we use the method from [10] and find values for the parameters and a steady state such that $\det(J_F(\phi))$ is negative. In this case

$$
\det(J_F(\phi)) = -T_2^2T_3T_7T_5z - r_1T_2T_7T_5z^2 - T_2T_3T_7T_5z^2 - T_2^2T_3T_7T_5z.
$$

Taking $r_1 = 1$ for $i = 1, \ldots, 5$ and $x_1 = 5, x_2 = 1, x_3 = 5$ this determinant is equal to -84. This means that the stoichiometric compatibility class containing $\phi(x)$ has more than one positive steady state. The total
amounts defining it are $T_1 = 30, T_2 = 1, T_3 = 180$. Using these parameters and solving either $F_T(x) = 0$ or $F_{T_1}(\varphi(z)) = 0$ we prove that there are three positive steady states in his stoichiometric compatibility class, as desired.

We conclude that the reduced network is bistable for every set of parameters for which there are three positive steady states, and the original network admits bistability in some region of the parameter space.

**Network (e).** In this network two substrates $S_0$ and $P_0$ are phosphorylated by the same kinase $E$, and dephosphorylated by the same phosphatase $F$.

\[ S_0 + E \xrightarrow{\kappa_1, \kappa_2} S_0E \xrightarrow{\kappa_3} S_1 + E \]
\[ S_1 + F \xrightarrow{\kappa_4, \kappa_5} S_1F \xrightarrow{\kappa_6} S_0 + F \]
\[ P_0 + E \xrightarrow{\kappa_7, \kappa_8} P_0E \xrightarrow{\kappa_9} P_1 + E \]
\[ P_1 + F \xrightarrow{\kappa_{10, \kappa_{11}}} P_1F \xrightarrow{\kappa_{12}} P_0 + F. \]

We rename the species as $E = X_1, F = X_2, S_0 = X_3, S_1 = X_4, P_0 = X_5, P_1 = X_6, S_0E = X_7, S_1F = X_8, P_0E = X_9, P_1F = X_{10}$, and their concentrations are denoted by lower case letters. For this network the polynomial $q_4(\lambda)$ has degree 6 and from the 6 Hurwitz determinants, the first three are positive for every positive steady state. The sign of $H_4$ is unclear and the analysis of the sign of $H_5$ was interrupted as the expanded computations could not be stored in a regular PC.

The positive steady state variety associated with this network can be parametrized by monomials as follows

\[ x_3 = \frac{\kappa_4\kappa_6(\kappa_2 + \kappa_3)}{\kappa_1\kappa_3(\kappa_5 + \kappa_6)} \cdot x_2 x_4 \]
\[ x_5 = \frac{\kappa_8 + \kappa_9}{(\kappa_1 + \kappa_2)\kappa_9\kappa_9} x_2 x_6 \]
\[ x_7 = \frac{\kappa_4\kappa_6}{\kappa_3(\kappa_5 + \kappa_6)} x_2 x_4 \]
\[ x_8 = \frac{\kappa_{10}\kappa_{12}}{\kappa_9(\kappa_{11} + \kappa_{12})} x_2 x_6 \]
\[ x_9 = \frac{\kappa_4}{\kappa_5 + \kappa_6} x_2 x_4 \]
\[ x_{10} = \frac{\kappa_{10}}{\kappa_{11} + \kappa_{12}} x_2 x_6. \]

With the notation of the manuscript, this parametrization $\phi$ corresponds to

\[ \alpha = \left( 1, 1, \frac{\kappa_4\kappa_6(\kappa_2 + \kappa_3)}{\kappa_1\kappa_3(\kappa_5 + \kappa_6)}, \frac{(\kappa_8 + \kappa_9)\kappa_{10}\kappa_{12}}{(\kappa_1 + \kappa_2)\kappa_9\kappa_9}, 1, \frac{\kappa_4\kappa_6}{\kappa_3(\kappa_5 + \kappa_6)}, \frac{\kappa_{10}\kappa_{12}}{\kappa_9(\kappa_{11} + \kappa_{12})}, \frac{\kappa_4}{\kappa_5 + \kappa_6}, \frac{\kappa_{10}}{\kappa_{11} + \kappa_{12}} \right) \]

\[ B = \begin{bmatrix}
1 & 0 & -1 & 0 & -1 & 0 & 0 & 0 & 0 & 0 \\
0 & 1 & 1 & 0 & 1 & 0 & 1 & 1 & 1 & 1 \\
0 & 0 & 1 & 1 & 0 & 0 & 1 & 0 & 1 & 0 \\
0 & 0 & 0 & 0 & 1 & 1 & 0 & 1 & 0 & 1
\end{bmatrix} \quad \text{and} \quad \xi = (x_1, x_2, x_4, x_6). \]

Using the identification between monomials and tuples it was possible for us to compute $H_4(\phi)$ and $H_5(\phi)$ and, after studying the signs of their coefficients, we have that $H_4(\phi)$ is positive but the sign of $H_5(\phi)$ is still unclear.

We proceed to **reduce the network** by removing all the reverse reactions and the intermediates $S_0E$ and $P_1F$. That is, the reactions $S_0 + E \rightarrow S_0E \rightarrow S_1 + E$ and $P_0 + F \rightarrow P_1F \rightarrow P_0 + F$ become $S_0 + E \rightarrow S_1 + E$ and $P_0 + F \rightarrow P_0 + F$ respectively. The reduced network is

\[ S_0 + E \xrightarrow{\kappa_1} S_1 + E \]
\[ S_1 + F \xrightarrow{\kappa_2} S_1F \xrightarrow{\kappa_3} S_0 + F \]
\[ P_0 + E \xrightarrow{\kappa_4} P_0E \xrightarrow{\kappa_5} P_1 + E \]
\[ P_1 + F \xrightarrow{\kappa_6} P_0 + F. \]

The species are renamed as $E = X_1, F = X_2, S_0 = X_3, S_1 = X_4, P_0 = X_5, P_1 = X_6, S_1F = X_8, P_0E = X_9$ and their concentrations are denoted by lower case letters. The polynomial $q_4(\lambda)$ associated with this
network has degree 4. The Hurwitz determinants \( H_1, H_2 \) and \( H_3 \) are positive for every positive steady state. However, the sign of \( H_4 \) is unclear even after evaluating at the parametrization \( \phi \) below:

\[
\phi(x_1, x_2, x_4, x_6) = \left( x_1, x_2, \frac{\tau_2 x_4}{\tau_1 x_1}, x_4, \frac{\tau_6 x_2 x_6}{\tau_3}, x_6, \frac{\tau_6 x_2 x_4}{\tau_3}, \frac{\tau_2 x_4}{\tau_3} \right).
\]

We explore the possibility of using Theorem 2 to ensure bistability.

The conservation laws of the system are

\[
x_1 + x_8 = T_1, \quad x_2 + x_9 = T_2, \quad x_3 + x_4 + x_9 = T_3 \quad \text{and} \quad x_5 + x_6 + x_8 = T_4.
\]

Taking \( i_1 = 1, i_2 = 2, i_3 = 3, i_4 = 5 \) we define \( F_T(x) \) as in equation [3] in the manuscript. Furthermore, the solutions of \( F_T(\ell) = 0 \) for \( \ell \neq 2 \) can be positively parametrized in terms of \( z = x_5 \) as

\[
\varphi(z) = \left( \frac{T_1 \tau_5}{\tau_4 z + \tau_5}, \frac{-T_1 \tau_4 \tau_5}{\tau_6 b_1(z)} (\tau_4 z + \tau_5) + b_1(z), \frac{-\tau_1 \tau_3 \tau_5 \tau_6}{\tau_4 z + \tau_5} b_2(z), \frac{-\tau_4 T_1 \tau_5}{\tau_4 z + \tau_5} b_2(z), \frac{-\tau_4 T_1 \tau_5}{\tau_4 z + \tau_5} b_2(z) \right)
\]

where

\[
b_1(z) := \tau_4 z^2 + ((T_1 - T_4) \tau_4 + \tau_5) z - T_4 \tau_5,
\]

\[
b_2(z) := \left( -\tau_1 \tau_3 \tau_4 \tau_5 + \tau_5 \tau_4^2 \right) z^2 + (T_1 \tau_1 \tau_2 \tau_4 \tau_5 - T_1 \tau_1 \tau_3 \tau_4 \tau_6 + T_4 \tau_1 \tau_3 \tau_4 \tau_6 - \tau_1 \tau_3 \tau_5 \tau_6 + \tau_2 \tau_3 \tau_4 \tau_5) z
\]

\[
+ T_4 \tau_1 \tau_3 \tau_5 \tau_6.
\]

Here \( \mathcal{E} = (0, \beta_1) \) where \( \beta_1 \) is the positive root of \( b_1(z) \), such that \( b_1(z) < 0 \) and \( b_2(z) > 0 \) in \( \mathcal{E} \).

The positive steady states in the stoichiometric compatibility class are in one to one correspondence with the roots of \( F_{T,2}(\varphi(z)) \). This is a rational function whose numerator is a polynomial of degree four with four independent term and the denominator is positive in \( \mathcal{E} \). Additionally, \( \varphi_2(z) = z \) and \( \varphi_3^2(z) = 1 \) \( \neq 0 \) and taking \( i = 5, j = 2 \) we have all the elements in the statement of Theorem 2. From the analysis of the Hurwitz determinants we know that the second hypothesis in the theorem holds. We now compute the relevant determinant to verify the first hypothesis:

\[
det(J_F(\varphi(z)))_{J,I} = \frac{-T_1 \tau_5 b_2(z)}{\tau_4 z + \tau_5}.
\]

This determinant is negative for every \( z \in \mathcal{E} \). We are then in the setting of Theorem 2. The sign of \( (F_{T,2} \circ \varphi)'(z_1) \), for \( z_1 \) the first root of \( (F_{T,2} \circ \varphi)(z_1) \), is positive as the independent term of \( (F_{T,2} \circ \varphi)(z) \) is negative. Hence, the sign of

\[
-(-1)^{5i+2}(1) = -1,
\]

which is positive. Thus, we conclude that the steady states \( \varphi(z_1), \varphi(z_2) \) are exponentially stable and \( \varphi(z_2) \ldots \) are unstable.

It is left to see that there exists a set of parameters such that the stoichiometric compatibility class has three positive steady states. In order to do so, we use the method from [10] and search for parameters and values of the concentration variables such that \( \det(J_F(\phi)) \) is negative:

\[
det(J_F(\phi)) = \tau_1 \tau_2 \tau_4 \tau_6 x_2 x_3^2 + \tau_1 \tau_2 \tau_4 \tau_6 x_1 x_2 x_4 + (\tau_2 \tau_5 + \tau_3 \tau_6) \tau_1 \tau_4 \tau_2 x_2 + \tau_1 \tau_2 \tau_3 \tau_5 x_1^2 x_4 + \tau_1 \tau_3 \tau_5 \tau_4 x_1^2
\]

\[
\quad + (\tau_1 \tau_5 + \tau_3 \tau_4) \tau_2 \tau_6 x_4 x_5^2 + \tau_1 \tau_2 \tau_3 \tau_6 \tau_1 x_2 x_4 + (\tau_1 \tau_5 + \tau_3 \tau_4) \tau_1 \tau_3 x_1 x_2 + \tau_1 \tau_2 \tau_5 x_2 x_6 + \tau_2 \tau_3 \tau_5 x_6^2
\]

\[
- \left( \frac{\tau_2}{\tau_1} - \frac{\tau_6}{\tau_5} \right) \tau_1 \tau_2 \tau_4 \tau_6 x_2 x_4 x_5^2 + \tau_1 \tau_2 \tau_5 x_6 x_2^2 + \tau_2 \tau_3 \tau_5 x_6^2
\]

Taking \( \tau_3 = 1, \tau_5 = 1, \tau_1 = 1, \tau_4 = 1, \tau_2 = 2, \tau_6 = 1 \) and \( x_1 = 1, x_2 = 1, x_4 = 9, x_6 = 9 \) the value of the determinant is -48. This implies that the stoichiometric compatibility class containing \( \phi(1,1,9,9) \) has more than one positive steady states and, solving either \( F_T(x) = 0 \) or \( F_{T,2}(\varphi(z)) = 0 \) we verify that it has three positive steady states.

We conclude that the reduced network is bistable for every set of parameters for which there are three positive steady states, and the original network admits bistability in some region of the parameter space.
Network (f). $E$ corresponds to a kinase that exists in two conformations: $E_1$ (relaxed state) and $E_2$ (tensed state). Each conformation acts as a kinase for a common substrate $S_0$. We denote by $S_1$ the phosphorylated form of the substrate. We assume that the intermediate kinase-substrate complexes, $E_1S_0$ and $E_2S_0$, also undergo conformational change.

$$
E_1 + S_0 \xrightarrow{\kappa_1} E_1S_0 \xrightarrow{\kappa_3} E_1 + S_1 \\
E_2 + S_0 \xrightarrow{\kappa_4} E_2S_0 \xrightarrow{\kappa_6} E_2 + S_1 \\
E_1 \xrightarrow{\kappa_8} E_2 \\
E_1S_0 \xrightarrow{\kappa_{10}} E_2S_0 \\
S_1 \xrightarrow{\kappa_7} S_0.
$$

The species are renamed as $E_1 = X_1, E_2 = X_2, E_1S_0 = X_3, E_2S_0 = X_4, S_0 = X_5, S_1 = X_6$ and their concentrations are denoted by lower case letters. The polynomial $q_\kappa(\lambda)$ associated with this network has degree 4. After computing and evaluating the Hurwitz determinants at a positive parametrization $\phi$ of the positive steady state variety, the sign of $H_3$ and $H_4$ is unclear. Since the hypotheses of Theorem 2 do not hold, we reduce the network by removing the intermediate $E_2S_0$ and all the reverse reactions. When removing $E_2S_0$ the reactions $E_2 + S_0 \xrightarrow{=} E_2S_0 \xrightarrow{=} E_2 + S_1$ become $E_2 + S_0 \xrightarrow{=} E_2 + S_1$ and the path $E_2 + S_0 \xrightarrow{=} E_2S_0 \xrightarrow{=} E_1S_0$ is collapsed to $E_2 + S_0 \xrightarrow{=} E_1S_0$. After removing the reverse reactions the reduced network is

$$
E_1 + S_0 \xrightarrow{\tau_1} E_1S_0 \xrightarrow{\tau_2} E_1 + S_1 \\
E_2 + S_0 \xrightarrow{\tau_3} E_2 + S_1 \\
E_2 + S_0 \xrightarrow{\tau_6} E_1S_0 \\
E_1 \xrightarrow{\tau_5} E_2 \\
S_1 \xrightarrow{\tau_4} S_0.
$$

The species are renamed as $E_1 = X_1, E_2 = X_2, E_1S_0 = X_3, S_0 = X_5, S_1 = X_6$ and their concentrations are denoted by lower case letters. We have $s = 3$. For this reduced network, $q_\kappa(\lambda)$ has degree 3 and the determinants $H_1, H_2$ are positive for every positive steady state. This means that the stability of steady states depends on the sign of $H_3$. We explore the possibility of applying Theorem 2 to ensure bistability. The conservation laws of the network are

$$x_1 + x_2 + x_3 = T_1 \quad \text{and} \quad x_3 + x_5 + x_6 = T_2.$$  

Taking $i_1 = 1$ and $i_2 = 3$ we define $F_T(x)$ as in equation [3] from the manuscript. The solutions of $F_{T,\ell}(x) = 0$ for $\ell \neq 3$ can be parametrized in terms of $z = x_5$ as

$$
\varphi(z) = \left(\frac{1}{b(z)} + \frac{T_1T_2\tau_6\tau_7\tau_9}{b(z)} : \frac{T_1\tau_6(\tau_7 + \tau_9 + \tau_7\tau_9)}{b(z)} , \frac{T_1\tau_2(\tau_1 + \tau_2 + \tau_1\tau_2)}{b(z)} , \frac{T_1\tau_2\tau_6(\tau_1 + \tau_2 + \tau_1\tau_2)}{b(z)} , \frac{T_1\tau_2\tau_6\tau_7\tau_9}{b(z)} \right)
$$

with $b(z) = \tau_1\tau_6z^2 + (\tau_2 + \tau_6)\tau_6z + \tau_2\tau_6$, and this parametrization is positive for every $z \in \mathbb{R}_{>0}$. Additionally $\varphi_4(z) = z$ and $\varphi(z) = 1 \neq 0$. With this parametrization, the positive steady states in one stoichiometric compatibility class are in one to one correspondence with the positive roots of $F_{T,3}(\varphi(x)) = 0$, that is a rational function whose numerator is a polynomial of degree 3 and has positive denominator:

$$F_{T,3}(\varphi(x)) = \frac{1}{(\tau_1\tau_6z^2 + \tau_2\tau_6z + \tau_6\tau_7\tau_9\tau_1)} \left[ (T_1\tau_1\tau_2\tau_6 + T_1\tau_1\tau_4\tau_6 - T_2\tau_1\tau_4\tau_6 + \tau_2\tau_4\tau_6 + \tau_4\tau_6\tau_9)z^2 + (T_1\tau_2\tau_6\tau_7\tau_9 + T_2\tau_2\tau_6\tau_7\tau_9 + T_1\tau_4\tau_6\tau_7\tau_9 - T_2\tau_2\tau_4\tau_6\tau_7\tau_9 - T_2\tau_2\tau_4\tau_6\tau_7\tau_9 + T_2\tau_4\tau_6\tau_7\tau_9 - T_2\tau_4\tau_6\tau_7\tau_9) \right].$$

Here $i = 4, j = 3$. After studying the Hurwitz determinants we know that the second hypothesis of Theorem 2 holds. We now check the sign of $\det(J_F(\varphi(z), T, J, I))$ on the first hypothesis of Theorem 2.

$$\det(J_F(\varphi(z), T, J, I)) = \tau_4 b(z),$$

29
which is positive for every $z \in \mathcal{E}$. We are now in the setting of Theorem 2. The sign of $(F_{T,3} \circ \varphi)'(z_1)$, for $z_1$ the first positive root of $F_{T,3}(\varphi(z))$, is positive as the independent term of $(F_{T,3} \circ \varphi)(z)$ is negative. Furthermore, the sign of
\[
\frac{(-1)^{s+i+j}}{\varphi_i'(z_1)}(F_{T,3} \circ \varphi)'(z_1) \det(J_{F_T}(\varphi(z_1)),J_{II})
\]
is $(-1)^{3+3+4}(1)(1) = 1$ positive, and the steady states $\varphi(z_1), \varphi(z_3), \ldots$ are exponentially stable and $\varphi(z_2), \ldots$ are unstable.

It remains to see that there exists a set of parameters such that the stoichiometric compatibility class has three positive steady states. In order to do this we use the method proposed in [10] and find values for the parameters and concentration variables such that $\det(J_F(\phi))$ is negative for the parametrization $\phi$ given as:
\[
\phi(x_2, x_5) = \left(\frac{\tau_6 x_2 x_5}{\tau_5}, x_2, \frac{\tau_6 x_2 x_5 (\tau_1 x_5 + \tau_5)}{\tau_2 \tau_5}, x_5, \frac{x_2 x_5 (\tau_1 \tau_6 x_5 + \tau_3 \tau_5 + \tau_5 \tau_6)}{\tau_4 \tau_5}\right).
\]
We obtain
\[
\det(J_F(\phi)) = \tau_1 (\tau_2 \tau_5 - \tau_3 \tau_5 + \tau_4 \tau_5) \tau_6 x_2 x_5^2 + 2(\tau_2 + \tau_4) \tau_1 \tau_5 \tau_6 x_2 x_5 + (\tau_2 \tau_3 + \tau_2 \tau_6 + \tau_4 \tau_6) \tau_5^2 x_2
\]
\[
+ \tau_1 \tau_4 \tau_5 \tau_6 x_5^2 + (\tau_2 + \tau_3) \tau_4 \tau_5 \tau_6 x_5 + \tau_2 \tau_4 \tau_5^2.
\]
Taking $\tau_i = 1$ for $i = 1, \ldots, 6$ and $x_2 = 2, x_5 = 6$ the value of the determinant is $-35$. This means that the stoichiometric compatibility class containing $\phi(x)$ has three positive steady states. The total amounts defining this class are $T_1 = 98, T_2 = 222$ and solving $F_T(x) = 0$ or $F_{T,3}(\varphi(z)) = 0$ we verify that there are three positive steady states.

We conclude that the reduced network is bistable for every set of parameters for which there are three positive steady states, and the original network admits bistability in some region of the parameter space.